Comparative Neuroanatomy of Mollusks and Nemerteans in the Context of Deep Metazoan Phylogeny

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Simone Faller

aus Frankfurt am Main

Berichter:
Privatdozent Dr. Rudolf Loesel
Universitätsprofessor Dr. Peter Bräunig

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1 General Introduction

Deep Metazoan Phylogeny

The concept of phylogeny follows directly from the theory of evolution as published by Charles Darwin in *The origin of species* (1859). According to this theory contemporary species share a common history through their ancestry. In the decades following 1859 first attempts for understanding the evolutionary history and reconstructing the phylogenetic relationships among animals were based on morphological comparisons. This approach lasted until the late 20th century when molecular methods advent and changed the traditional view on the animal tree of life (Fig. 1.1). The so-called "new animal phylogeny" (Adoutte et al. 2000) was initially based on the analysis of the nuclear small ribosomal subunit (18S) gene and rearranged the Bilateria into three clades: Deuterostomia, Lophotrochozoa, and Ecdysozoa (Fig. 1.2). The clade Lophotrochozoa, comprising annelids, mollusks, and the lophophorate phyla, was first introduced by Halanych et al. (1995). Shortly after, Aguinaldo et al. (1997) proposed the clade Ecdysozoa containing arthropods and other molting animals. The most prominent discrepancy resulting from this classification is the relative position of annelids and arthropods. Based on morphological properties, annelids and arthropods were grouped together in a single clade called Articulata. In contrast, molecular studies place annelids and arthropods into the different superphyla Lophotrochozoa and Ecdysozoa. Consequently, the "new animal phylogeny" was disputed by many morphologists (Wägele et al. 1999; Wägele & Misof 2001; Scholtz 2002). In addition, several multigene analyses failed to find support for the "new animal phylogeny" (Blair et al. 2002; Dopazo et al. 2004; Rogozin et al. 2007). However, recent phylogenomic studies using a multitude of species have confirmed the "new animal phylogeny" (Philippe et al. 2005; Helmkampf et al. 2008; Dunn et al. 2008). Despite this corroboration for grouping protostomes into Lophotrochozoa and Ecdysozoa, the relationships within these two superphyla vary strongly between different molecular analyses. Thus, even 150 years after *The origin of species* (Darwin 1859) the phylogenetic relationships of most major animal groups are still controversial. Therefore morphological characters are still needed as an independent approach to verify the molecular data.

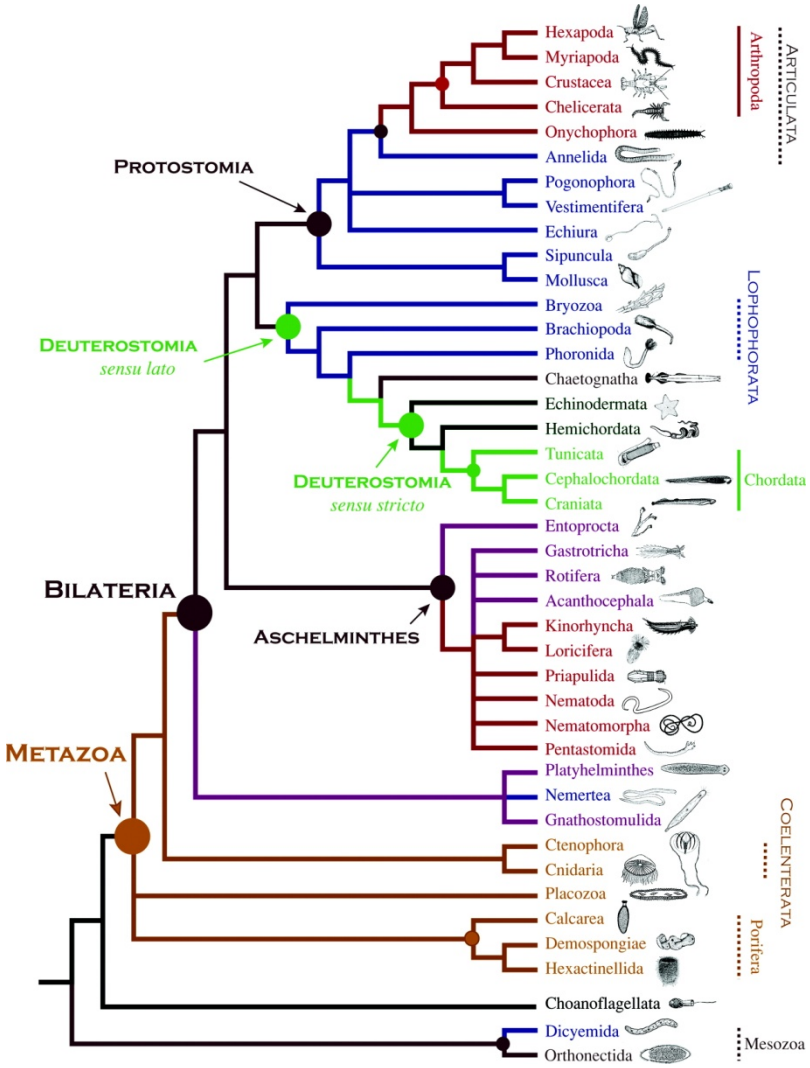

Figure 1.1 – The traditional view of animal phylogeny. The phylogenetic tree illustrates major concepts that are based on the analysis of morphological data. From Halanych (2004).

Neurophylogeny

In this approach one structure promising a multitude of morphological characters is the nervous system. The relevance of neuroanatomical characters for the inference of phylogenetic relationships was already investigated in the beginning of the 20th century by Nils Holmgren (1916) and his pupil Bertil Hanström (1928). They were among the first to characterize the internal brain anatomy of numerous invertebrate taxa, especially of arthropods, and thus added fundamental knowledge in arthropod evolution. However, in some extends their descriptions were rather superficial and the number and quality of original data presented unsatisfactory. Due to methodological advancements like immunohistochemistry and confocal laser scanning microscopy the field of comparative neuroanatomy has regained new impulses during the past decade and is now often referred to as “neurophylogeny” (Paul 1989; Harzsch 2002; Harzsch 2006). In addition to the technical

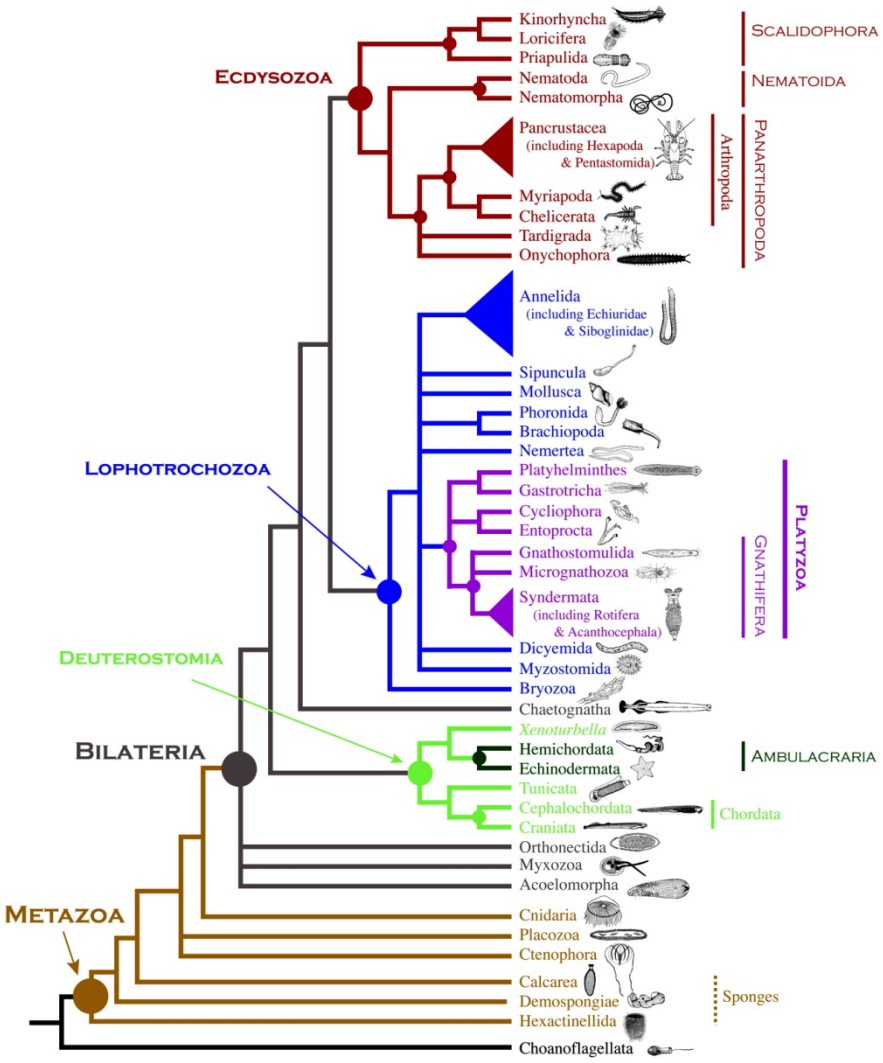

Figure 1.2 – The "new animal phylogeny". The phylogenetic tree is based on molecular data and illustrates the classification of Bilateria into Deuterostomia, Lophotrochozoa, and Ecdysozoa. From Halanych (2004).

progress, the methodological background for this discipline mainly relies on the foundation laid out by Kutsch and Breidbach (1994) who established criteria for comparing neuroanatomical characters between different species of arthropods. Based on these criteria, the nervous system has already been used extensively and as well successfully for the inference of phylogenetic relationships within the arthropods (Strausfeld 1998; Harzsch & Waloszek 2000; Loesel et al. 2002; Strausfeld et al. 2006a; Strausfeld & Andrew 2011). In addition, neuroanatomical data can also be utilized in a second way. By mapping neuroanatomical characters on trees that are generally accepted the evolution of particular structures of the nervous system can be retraced.

Arthropoda is the largest phylum of invertebrates and therefore it is not surprising that the amount of literature on the brain anatomy of this group is vast, first and foremost that of insects. In addition, the brain of arthropods provides a wealth of morphological features.

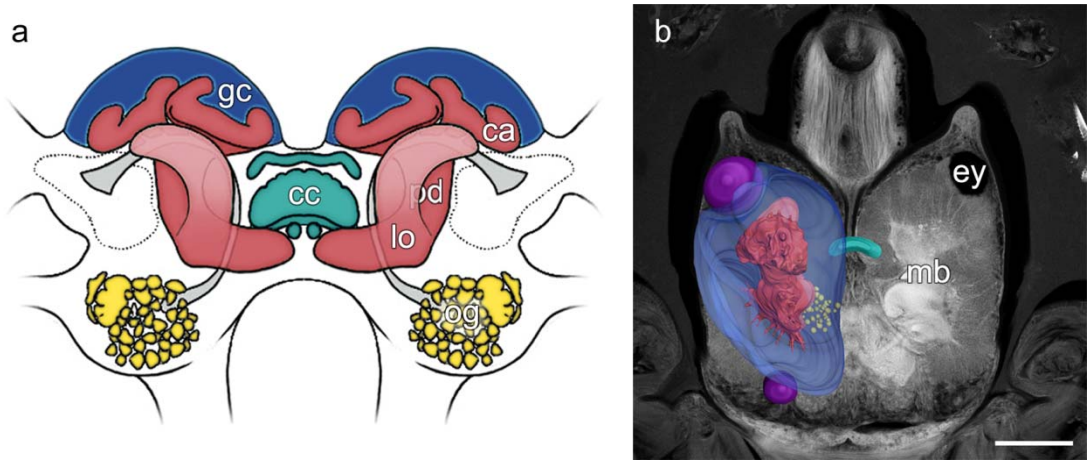

Figure 1.3 – Architecture of an insect (a) and an annelid (b) brain. a Schematic diagram showing the major neuropils of the insect brain. Modified from Strausfeld et al. (1998). **b** Three-dimensional surface reconstruction superimposed upon an autofluorescence image demonstrating that the annelid brain is composed of similar neuropils. From Heuer and Loesel (2009). *ca* calyx; *cc* central complex; *ey* eye; *gc* globuli cells; *lo* lobe; *mb* mushroom body; *og* olfactory glomeruli; *pd* peduncle. Scale bar: b = 200 µm.

Figure 1.3a displays the major neuropils of the insect brain: the paired mushroom bodies, the central complex (green), and the olfactory glomeruli (yellow). The most prominent of these neuropils are the mushroom bodies built by the ramifications of the so-called globuli cells that for historical reasons in insects are called Kenyon cells. The cell bodies of thousands of these neurons form a dense cluster that surrounds the input region of the mushroom bodies, the so-called calyces. The mushroom bodies receive multimodal sensory input and play a role in associative learning and memory formation (Heisenberg 2003; Campbell & Turner 2010). The mushroom bodies as well as the remaining neuropils are highly conserved and present basically in all arthropod groups, even in onychophorans (Strausfeld et al. 2006a; Strausfeld et al. 2006b).

In comparison to the vast amount of neuroanatomical studies on arthropods, analyses focusing on neuroanatomical characters in non-arthropod protostome phyla are rare. However, a recent neuroanatomical study on annelids (Heuer 2010) demonstrates that the brain of polychaete annelids is composed of similar neuropils as the arthropod brain (cf. Fig. 1.3a, b). Moreover, the most prominent neuropil of the arthropod brain, the mushroom bodies are built just in the same way in polychaete annelids, implying a possible homology of arthropod and annelid mushroom bodies. Recently, the morphological-derived homology assumption has been corroborated by molecular fingerprint studies, providing as well strong evidence for a homology of insect and annelid mushroom bodies (Tomer et al. 2010).

In the light of the "new animal phylogeny" the homology of arthropod and annelid mushroom bodies implies that these structures have to be a plesiomorphic character trait of all protostomes. Since comparably well-developed mushroom bodies have not yet been identified in any other protostome clade, the homology of arthropod and annelid mushroom bodies requires a secondary reduction of these neuropils in almost all protostome taxa.

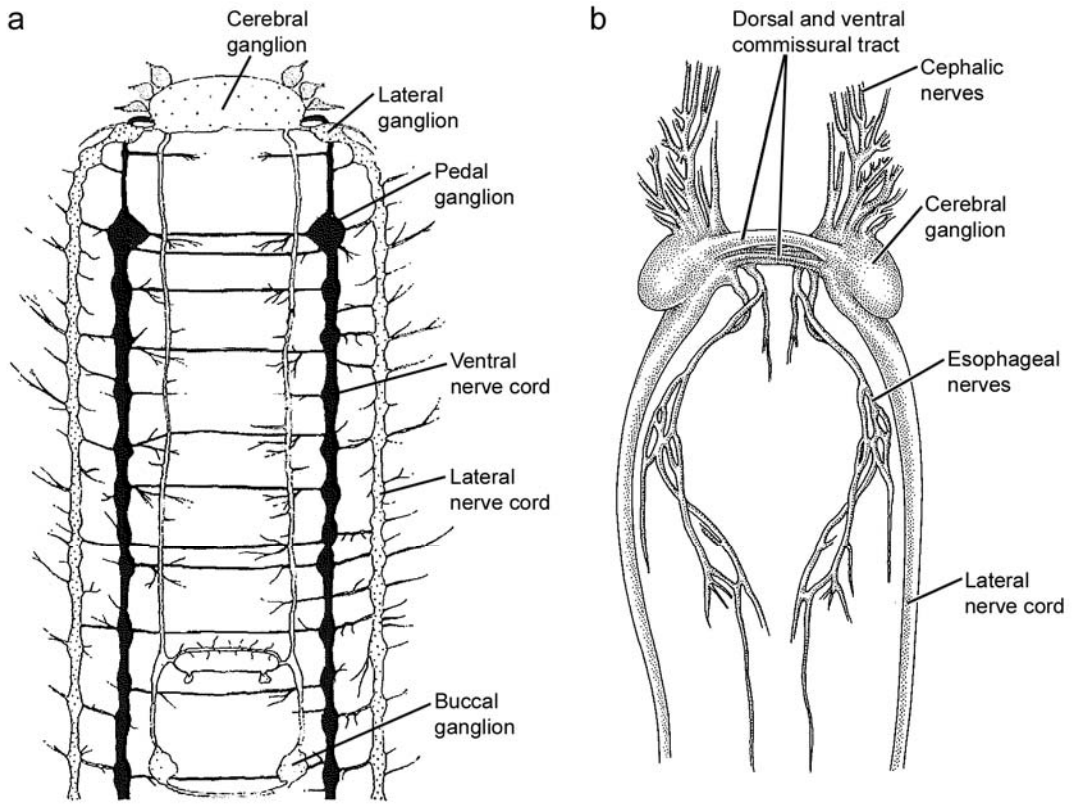

Figure 1.4 – Neuroarchitecture of a non-conchiferan mollusk (a) and a nemertean (b) representative. a Schematic diagram showing the major components of the anterior nervous system of *Syngenoherpia intergenerica* (Solenogastres, Mollusca). Modified from Salvini-Plawen (1972). **b** Schematic diagram showing the major components of the anterior nervous system of *Tubulanus allunatus* (Anopla, Nemertea). Modified from Brusca and Brusca (2003).

However, one should consider that neuroanatomical studies of the majority of non-arthropod animals were frequently focused on the gross anatomy rather than on its internal structure (c.f. Fig. 1.4a, b). Therefore in-depth investigations of the neuroanatomy of further protostome clades are absolutely essential to verify whether higher brain centers like the mushroom bodies are really not detectable in other protostome taxa, especially in those which possess a well developed brain.

Mollusca

The metazoan phylum Mollusca is the second largest after Arthropoda comprising about 130,000 extant species (Haszprunar et al. 2008) and a remarkable fossil record. With eight extant class-level taxa and an enormous variation in body plans Mollusca are one of the most diverse groups of animals. Despite this diversity, all living classes are predominantly marine. The major apomorphic characters unifying the phylum are the unique radula, the mantle, and the foot (Nielsen 2001).

Although mollusks were studied in some of the earliest zoological investigations, our knowledge about the molluscan evolutionary history is surprisingly incomplete. Albeit there is a growing acceptance that Mollusca are grouped within the clade Trochozoa, the kinship to other lophotrochozoan taxa is still a matter of debate. Aside from the unsettled position of the whole phylum, the classification of the eight molluscan taxa has been controversial since the very beginning of comparative investigations (Salvini-Plawen & Steiner 1996) and remains among the most challenging phylogenetic problems (Giribet et al. 2007). In general, the so-called higher Mollusca are unified as Conchifera, but grouping within this clade varies strongly between different studies (cf. Fig. 1.5a-f). Traditionally, the remaining non-conchiferan mollusks (Solenogastres, Caudofoveata, and Polyplacophora) are viewed as the most basal extant mollusk lineages (Salvini-Plawen 2003; Haszprunar et al. 2008; Todt et al. 2008b). In contrast, the most recent phylogenomic data place them as a sister group to the Conchifera (Kocot et al. 2011; Smith et al. 2011).

Even though members of the conchiferan Gastropoda and Cephalopoda belong to the best known invertebrates, the taxa with smaller numbers of species, especially the potentially basal taxa, were often neglected in morphological as well as in molecular investigations. While the nervous system of gastropod mollusks is well studied and has even been used for the inference of phylogenetic relationship within Gastropoda (Haszprunar 1988; Huber 1993), our standard of knowledge about the remaining taxa is remarkable low and the ancestral type of the nervous system is still unclear.

Nemertea

Compared to Arthropoda and Mollusca, Nemertea is only a minor metazoan phylum, comprising about 1280 species (Kajihara et al. 2008) grouped in two different class-level taxa. Members of Nemertea are vermiform unsegmented animals that are most abundant in marine environments, inhabiting a wide range of interstitial, benthic, or pelagic habitats. The majority of nemerteans are active predators. To catch and intoxicate their prey organisms they possess a unique structure, the eversible proboscis. The active and predatory lifestyle is also reflected in a complex nervous system with a cerebral ganglion that is formed of four interconnected lobes encircling the proboscis (Brusca & Brusca 2003).

Due to morphological characters, Nemertea were traditionally placed close to Platyhelminthes (Nielsen 2001). Even though none of the molecular based studies found support for this relationship, the placement of Nemertea within Lophotrochozoa varies between different studies (Turbeville & Smith 2007; Dunn et al. 2008; Struck & Fisse 2008; Hejnol et al. 2009; Paps et al. 2009; Podsiadlowski et al. 2009). According to a recent study focusing on the phylogenetic position of Nemertea using phylogenomic data, nemerteans are grouped together in a clade with annelids and mollusks (Struck & Fisse 2008).

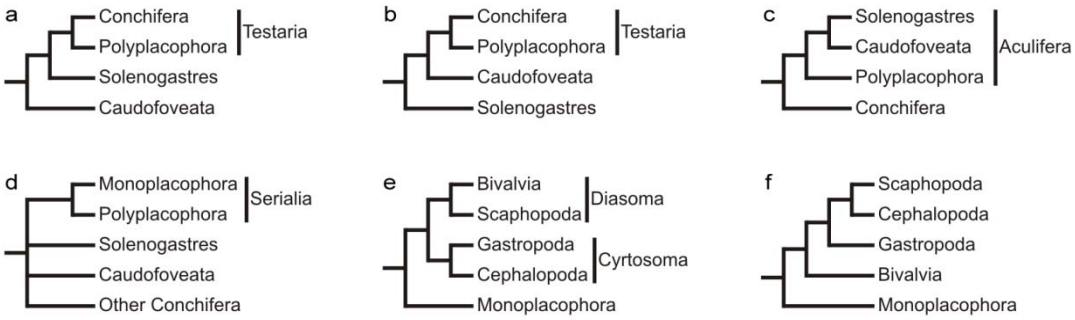

Figure 1.5 – Main hypotheses of molluscan phylogeny. a Adenopoda hypothesis placing Caudofoveata basal. **b** Hepagastralia hypothesis placing Solenogastres basal. **c** Aculifera hypothesis placing Aplacophora sister to Polyplacophora. **d** Serialia hypothesis uniting Polyplacophora and Monoplacophora. **e** Diasoma hypothesis uniting Bivalvia and Scaphopoda. **f:** Helcionellid hypothesis uniting Scaphopoda and Cephalopoda. Redrawn from Kocot et al. (2011).

Aim of the thesis

This doctoral thesis is embedded in the priority program *Deep Metazoan Phylogeny* that aims to reconstruct the evolutionary history and the phylogenetic relationships among animals with molecular as well as morphological methods. Prior to *Deep Metazoan Phylogeny*, the amount of neuroanatomical data was only low for the majority of non-arthropod invertebrates and the comparability of available data was limited due to different methodological approaches. Therefore the working groups investigating morphological features largely concentrate on adding new neuroanatomical characters for so far poorly investigated invertebrate taxa. Moreover using a pre-defined set of immunohistochemical markers a compatibility of the received data is assured. As a part of the *Deep Metazoan Phylogeny* program, this doctoral thesis concentrates on adding new neuroanatomical data for two important lophotrochozoan phyla: Mollusca and Nemertea.

In particular the present thesis aims to answer the following questions:

- Does the brain of mollusks and nemerteans contain clearly defined subcompartments?
- Does the brain of mollusks offer particular structures that occur only in defined molluscan taxa? If this is the case, is there a phylogenetic signal for the classification of mollusks?
- Is any of the neuropils in the molluscan or nemertean brain comparable to the major neuropils found in the arthropod and annelid brain?
- Is there a deep-time origin of neuroanatomical features?

2 Neuroanatomy of Minor Mollusca

Introduction

The structure of the nervous system has always provided strong and important arguments in the discussion on metazoan phylogeny (Hanström 1928; Loesel 2011). In recent years, the field of comparative neuroanatomy regained new impulses by methodological advances, like immunohistochemistry and confocal laser scanning microscopy, and is now often referred to as "neurophylogeny" (Paul 1989; Harzsch 2002; Harzsch 2006). The majority of the prevailing neurophylogenetic studies have been restricted to arthropods (Strausfeld 1998; Loesel et al. 2002; Loesel 2005; Strausfeld & Andrew 2011).

Next to arthropods, mollusks are the second largest phylum of invertebrates and exhibit an enormous diversity of body plans. While neuroanatomical studies on gastropods and cephalopods are numerous, little is known about the remaining classes (Caudofoveata, Solenogastres, Polyplacophora, Monoplacophora, Bivalvia, and Scaphopoda). Here, the aplacophoran taxa (Solenogastres and Caudofoveata) and Polyplacophora are of particular interest because they supposedly constitute the most basal extant mollusk lineages (Salvini-Plawen 2003; Haszprunar et al. 2008; Todt et al. 2008b), or alternatively a sister group to the remaining mollusks, the Conchifera (most recently: Kocot et al. 2011; Smith et al. 2011; Vinther et al. 2011). In addition, our standard of knowledge about aplacophoran mollusks is especially low because of the great difficulties to collect them (Salvini-Plawen 2003). While the monophyly of Conchifera is generally accepted (Haszprunar et al. 2008), the interrelationships between the remaining classes are still ambiguous. Based on morphology, solenogasters and caudofoveates are often grouped together as Aplacophora (Hyman 1967; Scheltema 1993). But this grouping is also disputed by others (Salvini-Plawen 1972; Salvini-Plawen 1980; Salvini-Plawen 1981; Haszprunar 2000; Salvini-Plawen 2003). Recently, molecular phylogenies have been supporting the Aplacophora hypothesis (Kocot et al. 2011; Smith et al. 2011; Vinther et al. 2011). Both, solenogasters and caudofoveates are marine vermiform animals. Instead of a shell their body is covered by sclerites. The predatory solenogasters have a ciliated gliding sole (foot) located in a ventral furrow. In contrast, the micro-omnivorous caudofoveates do not possess a foot. Rather are these animals

distinguished by an oral shield that is used for burrowing. Polyplacophorans are likewise exclusively marine mollusks, characterized by eight dorsal shell plates.

In addition to the unsettled placement of the three non-conchiferan molluscan groups, most recent disagreements center around the systematic position of the conchiferan scaphopods (Glaubrecht et al. 2005; Kocot et al. 2011). Scaphopods are entirely marine infaunal mollusks. Within the phylum Mollusca they have been traditionally regarded as the sister taxon of the Bivalvia, together forming the clade Diasoma (Runnegar & Pojeta 1974). Early molecular research favored a close relationship of the Scaphopoda and Cephalopoda, i.e. the so-called helcionellid concept (Steiner & Dreyer 2003; Passamaneck et al. 2004), while recent phylogenomic studies place them either as a sister group to a clade formed by Bivalvia and Gastropoda (Pleistomollusca: Kocot et al. 2011) or a sister to Gastropoda (Smith et al. 2011). The analysis of an additional set of morphological characters might help to resolve these questions.

Neuroanatomical studies on aplacophoran mollusks are rare and immunocytochemical investigations have not been published until the last years by Shigeno et al. (2007) and Todt et al. (2008a). Because each of these studies focuses only on one species of Caudofoveata or Solenogastres, it is not clear how far they are valid for the whole group. Almost the same is true for polyplacophorans, where comparative studies are lacking as well. Virtually nothing is known about the detailed neuroanatomy of scaphopods and in general comparative neuroanatomical studies across the molluscan classes using the same staining techniques do not exist.

The present study therefore provides the first comparative immunohistochemical data on the nervous system of selected species of caudofoveates, solenogasters, polyplacophorans, and scaphopods.

Materials and Methods

The investigated animals were collected during field trips. A detailed list of investigated species, particular collection sides, and number of specimens is provided in table 2.1. The neuroarchitecture was revealed by a combination of immunohistochemistry and DAPI nuclear labeling as described in Heuer and Loesel (2008a). Depending on the size of the investigated species, the mollusks were either decapitated or fixed completely overnight in 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS) at 4°C. Following fixation the heads or whole animals were washed and stored in PBS.

For vibratome-sectioning, the caudofoveate, solenogaster, and polyplacophoran specimens were decalcified in 80% 0.23 M EDTA and 20% 0.1 M sodium acetate. The shells of the scaphopods were cracked and removed carefully. All samples were embedded in a gelatine/albumin medium and hardened overnight in 14% formalin in PBS at 4°C. The

Table 2.1 – List of investigated species, including sampling locality and number of investigated specimens.

Species	Class/ Family	Depth, Sampling locality	Investigated specimens
Acanthochitona crinita (PENNANT, 1777)	Polyplacophora/ Acanthochitonidae	Intertidal, Roscoff, France	4
Antalis entalis (LINNAEUS, 1758)	Scaphopoda/ Dentaliidae	90-200 m, Bergen, Norway	12
Dorymenia sarsii (KOREN AND DANIELSSEN, 1877)	Solenogastres/ Proneomeniidae	90-200 m, Bergen, Norway	5
Entalina quinquangularis (FORBES, 1843)	Scaphopoda/ Entalinidae	90-200 m, Bergen, Norway	5
Falcidens crossotus SALVINI-PLAWEN, 1968	Caudofoveata/ Chaetodermatidae	90-200 m, Bergen, Norway	8
Lepidochitona cinerea (LINNAEUS, 1767)	Polyplacophora/ Ischnochitonidae	Intertidal, List, Sylt, Germany	14
Scutopus ventrolineatus SALVINI-PLAWEN, 1968	Caudofoveata/ Limifossoridae	90-200 m, Bergen, Norway	16

next day, the gelatin/albumin blocks were cut with a vibratome (VT1000S, Leica Microsystems, Germany) into sections of 80 µm in thickness. After washing the sections six times in PBS with 0.1% Triton X-100 (TX) to increase membrane permeability they were preincubated overnight in PBS containing 0.5% TX and 5% normal swine serum (Jackson ImmunoResearch, West Grove, PA) as blocking reagent. The primary antibodies either anti-FMRF-amide (developed in rabbit; ImmunoStar, Hudson, WI) or alternatively anti-serotonin (developed in rabbit; Sigma-Aldrich, Saint Louis, MO) were added directly to this blocking solution in a dilution that varied from 1:2000 to 1:30000 depending on the incubation time of 28 h up to 45 h at room temperature. In selected species both primary antibodies were additionally used in a combination with anti acetylated α-tubulin (developed in mouse; Sigma-Aldrich, Steinheim, Germany) diluted 1:500. After washing the sections again six times in PBS with 0.1% TX they were then incubated overnight in secondary antibody conjugated to a fluorophore (Cy3-conjugated goat anti-rabbit and Cy2-conjugated goat anti-mouse; Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:2000 in PBS containing 0.5% TX and 1% normal swine serum. Following removing of the secondary antiserum, the cell nuclei were counterstained by incubating the sections with DAPI (4′,6-Diamidino-2-phenylindole, dilactate; Sigma-Aldrich, Steinheim, Germany) at a dilution of 1:1000 in PBS for 12 min. After another several washes in PBS containing 0.1% TX the

sections were finally mounted on chrome alum/gelatin coated glass slides and covered with glass coverslips by using Elvanol mounting medium (Rodriguez & Deinhardt 1960).

Preparations were viewed and photographed on a confocal laser scanning microscope (TCS SP2, Leica Microsystems, Germany). A helium/neon laser (excitation wavelength 543 nm, detection range 555-700 nm) was used to detect Cy3 fluorescence and an argon/krypton laser (excitation wavelength 488 nm, detection range 500-535 nm) was used to detect Cy2 fluorescence. DAPI fluorescence was detected with a diode laser (excitation wavelength 405 nm, detection range 410-550 nm). The resulting image stacks were collapsed using the "maximal projection" tool of the TCS SP2 Leica confocal software. Images were further processed with Adobe Photoshop utilizing global imaging enhancement tools (contrast, brightness).

Results

Immunohistochemical methods were used to analyze the nervous system of seven molluscan species belonging to four different taxa (see table 2.1). For each taxon investigated we first describe the pattern of the nervous system on the basis of one investigated species in detail. Afterwards similarities and differences are highlighted by comparing the respective patterns with a second species of each taxon, which belongs to another family. The data for both species are combined in a schematic drawing representing the main components of the anterior nervous system of Caudofoveata, Solenogastres, Polyplacophora, and Scaphopoda (Fig. 2.1). The nomenclature used for describing neuroanatomical structures in this study conforms to Richter et al. (2010).

Caudofoveata

Scutopus ventrolineatus

The brain of *S. ventrolineatus* is situated in the anterior dorsal part of the animal and occupies almost half of the head's width. It is composed of fused ganglia that in the posterior part are heterolaterally bipartite (Fig. 2.2a-d). Therefore the anterior posterior diameter of the brain varies between 30 µm in the median and up to 90 µm in the lateral parts.

FMRFamide-like immunoreactivity (-ir) demonstrates that the brain is composed of a central neuropil that is divided into an anterior and a posterior portion (see below). Apart from this, further subcompartments of the central neuropil are not discernable. FMRFamide-like immunoreactive somata reside laterally to the central fiber mass. Aside from the immunoreactivity in the brain FMRFamide-like-ir was also found within the buccal ganglia, the ventral and lateral nerve cords extending towards the posterior part of the body, and in

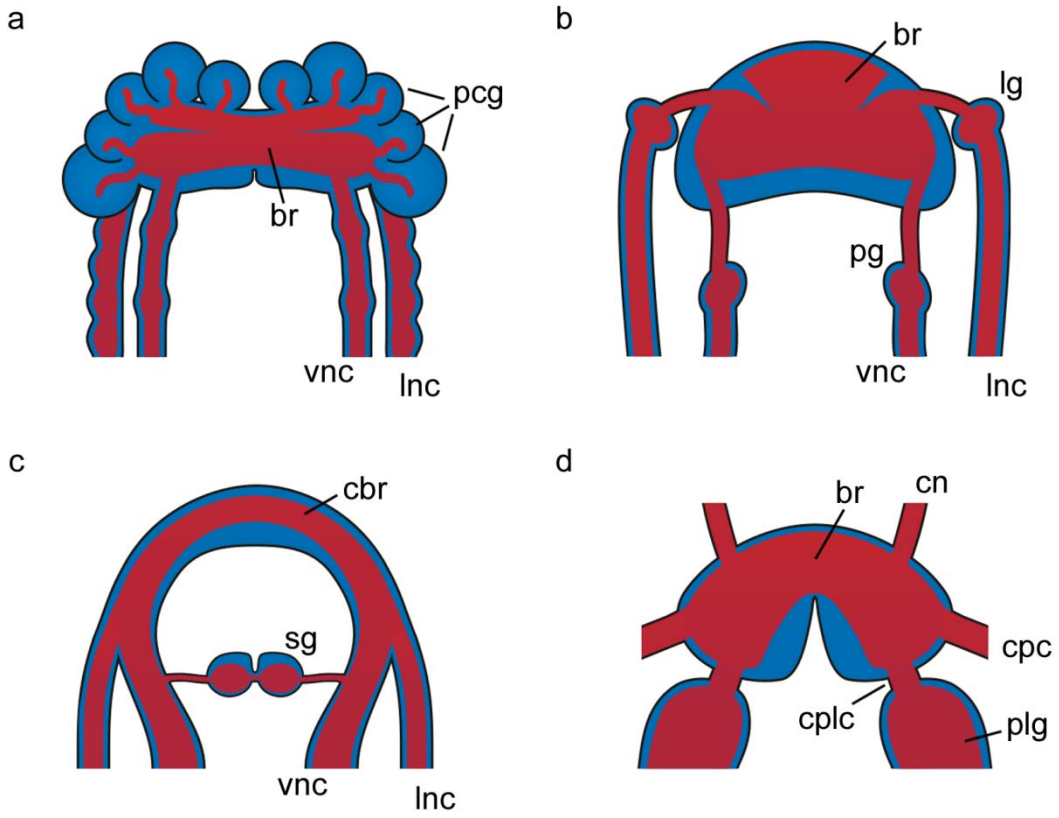

Figure 2.1 – Schematic diagrams of the brain and anterior nervous system of the four investigated molluscan taxa. a Caudofoveata, **b** Solenogastres, **c** Polyplacophora, and **d** Scaphopoda. Like in the original stainings immunoreactive fibers are illustrated in red and DAPI labeled nuclei are color-coded in blue. *br* brain; *cbr* cerebrobuccal ring; *cn* captacular nerves; *cpc* cerebropedal connective; *cplc* cerebropleural connective; *lg* lateral ganglion; *lnc* lateral nerve cord; *pcg* precerebral ganglia; *pg* pedal ganglion; *plg* pleural ganglion; *sg* subradular ganglion; *vnc* ventral nerve cord.

smaller fiber bundles proceeding into the anterior direction or forming a loop in front of the anterior ventral part of the brain (Fig. 2.2a, c).

The overlay images (Fig. 2.2b-g) demonstrate that the brain and the buccal ganglia are covered by neuronal somata. These neuronal somata build up a tightly packed cortex around the central neuropil. This cortex is most expanded at the posterior and more dorsal side of the brain. Here, it forms an equally thick layer in each hemisphere, which is interrupted in the center (Fig. 2.2b, c). Apart from the neuronal somata covering the brain, a band of neuronal somata divide the central neuropil into a minimum of two layers, an anterior third and a posterior portion (Fig. 2.2d, e, f, g). In addition, anterior to the brain neuronal somata form well-defined spherical shaped clusters (Fig. 2.2b, d, e, f, g). The shape and size of these so-called precerebral ganglia varied within the nervous system of one specimen as well as among different individuals (Fig. 2.2e-g). Moreover, also the number of definable clusters varied between different individuals. However, in most cases five clusters per hemisphere could be distinguished (Fig. 2.2d, e, f). The internal structure of each of these precerebral

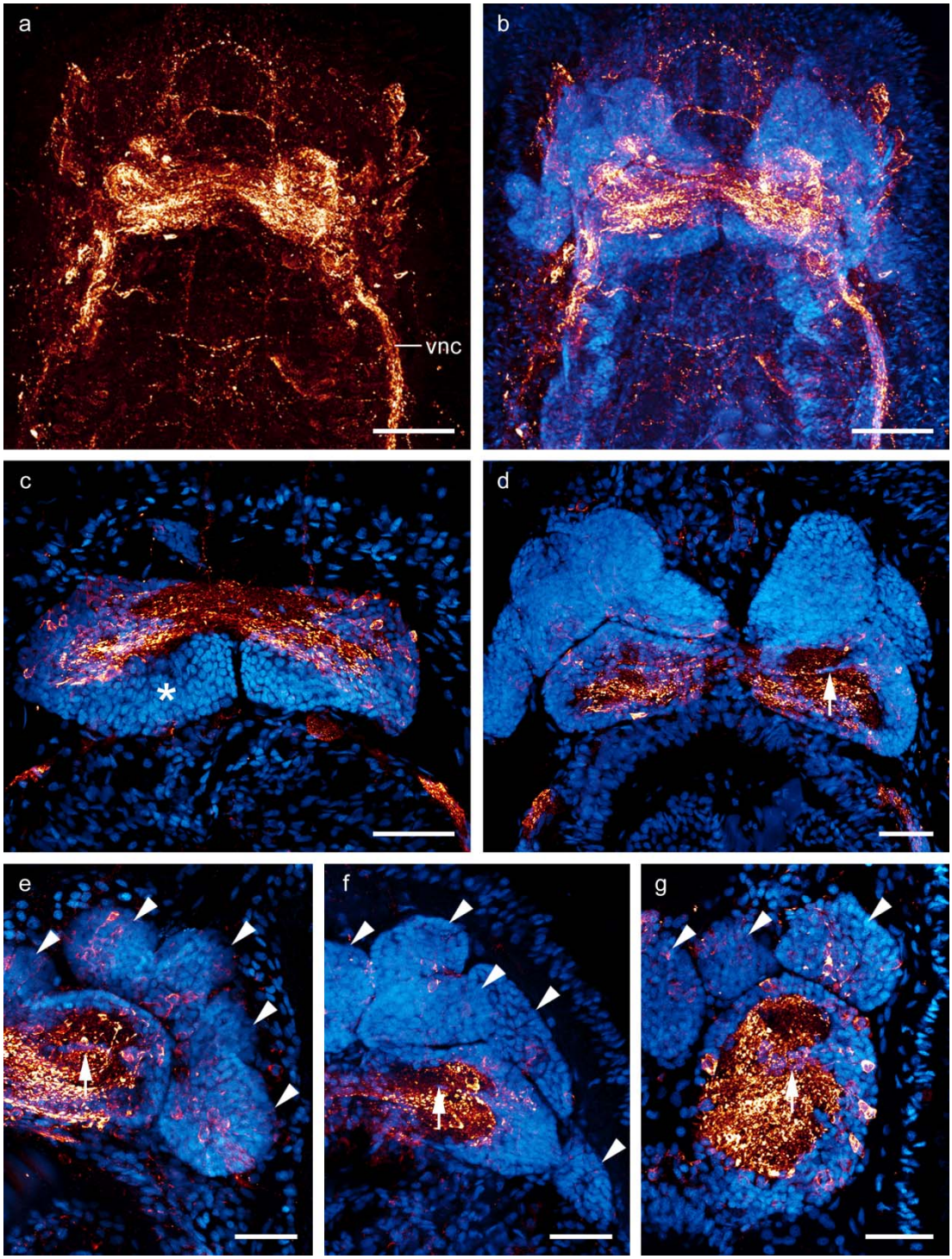

Figure 2.2 – FMRFamide-like immunoreactivity and DAPI nuclear labeling (blue) of the anterior nervous system of *Scutopus ventrolineatus.* a Reconstruction of the anterior nervous system composed by the overlay of six individual confocal images revealing the brain and the paired ventral nerve cord (*vnc*). **b** Superposition image of FMRFamide-like-ir and DAPI nuclear labeling showing the same detail as in **a**. **c-d** Higher magnification of the brain in two consecutive horizontal sections showing the thick layer of neuronal somata in the dorsal part (*asterisk* in **c**) as well as the precerebral ganglia and the band of neuronal somata (*arrow* in **d)** in the ventral part of the brain. **e-g** Higher magnification of one hemisphere of the brain showing the band of neuronal somata dividing the central neuropil (*arrow*) as well as the number, shape and size of the precerebral ganglia (*arrowheads*) in three different specimens. Scale bars: a,b = 80 µm; c,d,e,f,g = 40 µm.

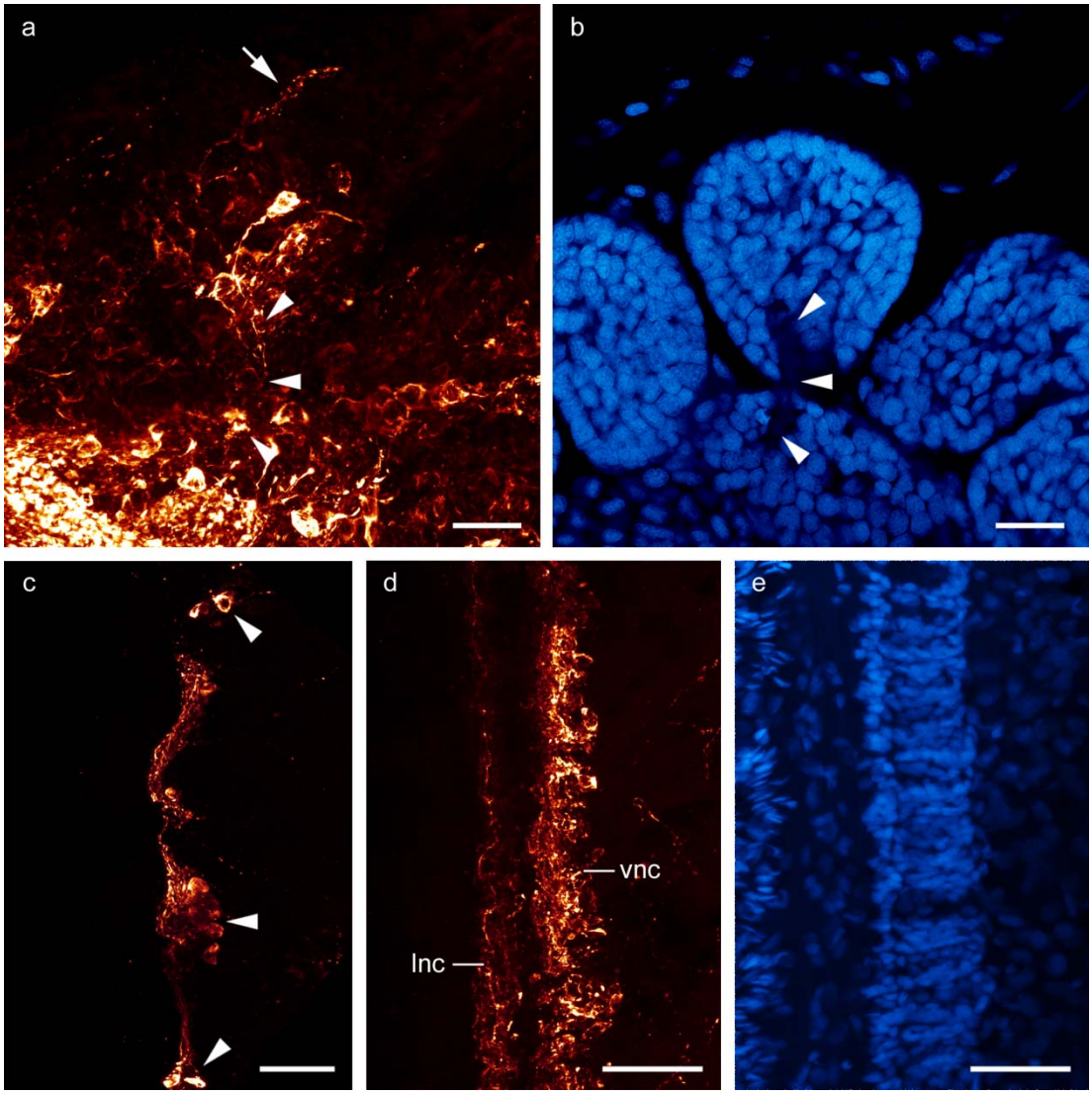

Figure 2.3 – Higher magnification of the precerebral ganglia and the nerve cords of *Scutopus ventrolineatus*. a FMRFamide-like-ir showing that the precerebral ganglia are built predominantly by somata that are connected to the brain (*arrowheads*) as well as to anterior parts of the body (*arrow*). **b** DAPI nuclear labeling of the same region as shown in **a**. **c** FMRFamide-like-ir of the lateral nerve cord in the head region showing that it forms ganglion-like swellings (*arrowheads*). **d** FMRFamide-like-ir in the trunk region demonstrating that the lateral (*lnc*) and ventral nerve cords (*vnc*) proceed close to each other. **e** DAPI nuclear labeling of the same region as shown in **d**. Scale bars: a,b = 20 µm; c,d,e = 40 µm.

ganglia is completely different to the constitution in the brain. The precerebral ganglia do not contain a central neuropil but are rather built primarily by somata that in part show FMRFamide-like-ir. These FMRFamide-like immunoreactive somata are connected to the central neuropil of the brain as well as to anterior parts of the body (Fig. 2.3a). In addition, the DAPI nuclear labeling confirms that the precerebral ganglia are built throughout by tightly packed neuronal somata that are only recessed in the area where fiber bundles connecting the precerebral ganglia to the brain (Fig. 2.3b).

Like the brain, the paired ventral and lateral nerve cords are built by a core neuropil that is covered by neuronal somata, characterizing them as medullary cords. Conspicuously, the core neuropil is not of equal diameter but shows serially repeated ganglion-like swellings that are most pronounced in the head region (Fig. 2.3c). More posteriorly, the lateral and ventral nerve cords proceed close to each other (Fig. 2.3d). Here, the neuronal somata form discrete packages along the ventral nerve cords (Fig. 2.3e).

Falcidens crossotus

In principle, the nervous system of *F. crossotus* corresponds to that of *S. ventrolineatus*. FMRFamide-like-ir was found within the brain, the buccal ganglia, and the ventral and lateral nerve cords (Fig. 2.4a). In addition, anti acetylated α-tubulin-ir show the innervation of the oral shield (Fig. 2.4b). The brain is built by fused ganglia that in the ventral part are still clearly bipartite (Fig. 2.4c). It is composed by external somata that surround a central neuropil. The DAPI nuclear labeling demonstrates that the layer of neuronal somata which surrounds the central neuropil is most pronounced in the anterior part of the brain (Fig. 2.4c, d). Furthermore, a band of neuronal somata penetrates the central neuropil and divides it into an anterior and a posterior portion. Like in *S. ventrolineatus*, neuronal somata form up to five clusters of precerebral ganglia per hemisphere (Fig. 2.4e). In addition, there is one structure connected with the brain of *F. crossotus*, which was not detected in the brain of *S. ventrolineatus*: In each hemisphere the central neuropil of the dorsal part of the brain is connected to a small cluster of serotonin-like immunoreactive somata, which is situated anterior to the brain neuropil (Fig. 2.4d).

Like in *S. ventrolineatus*, the ventral and lateral nerve cords are medullary cords characterized by neuropil that is covered by neuronal somata (Fig. 2.4a) and the DAPI nuclear labeling shows that the neuronal somata form discrete packages along the ventral nerve cords.

Solenogastres

Dorymenia sarsii

The most prominent structure of the nervous system of *D. sarsii* is a kidney shaped brain that spans almost half of the head's width and is situated dorsally just posterior to the pedal pit. In horizontal sections, the brain does not show any evidence of bisection and the two hemiganglia are completely fused (Fig. 2.5a-d).

The internal structure of the brain is characterized by different immunostaining patterns when comparing its ventral and its dorsal aspects. FMRFamide-like immunoreactive processes form a pronounced X-shaped structure at the dorsal part of the brain (Fig. 2.5a, b), but are more or less equally distributed at its ventral part (Fig. 2.5c, d). There, a commissural fiber tract spans the entire width of the brain (asterisk Fig. 2.5c). The overlay image

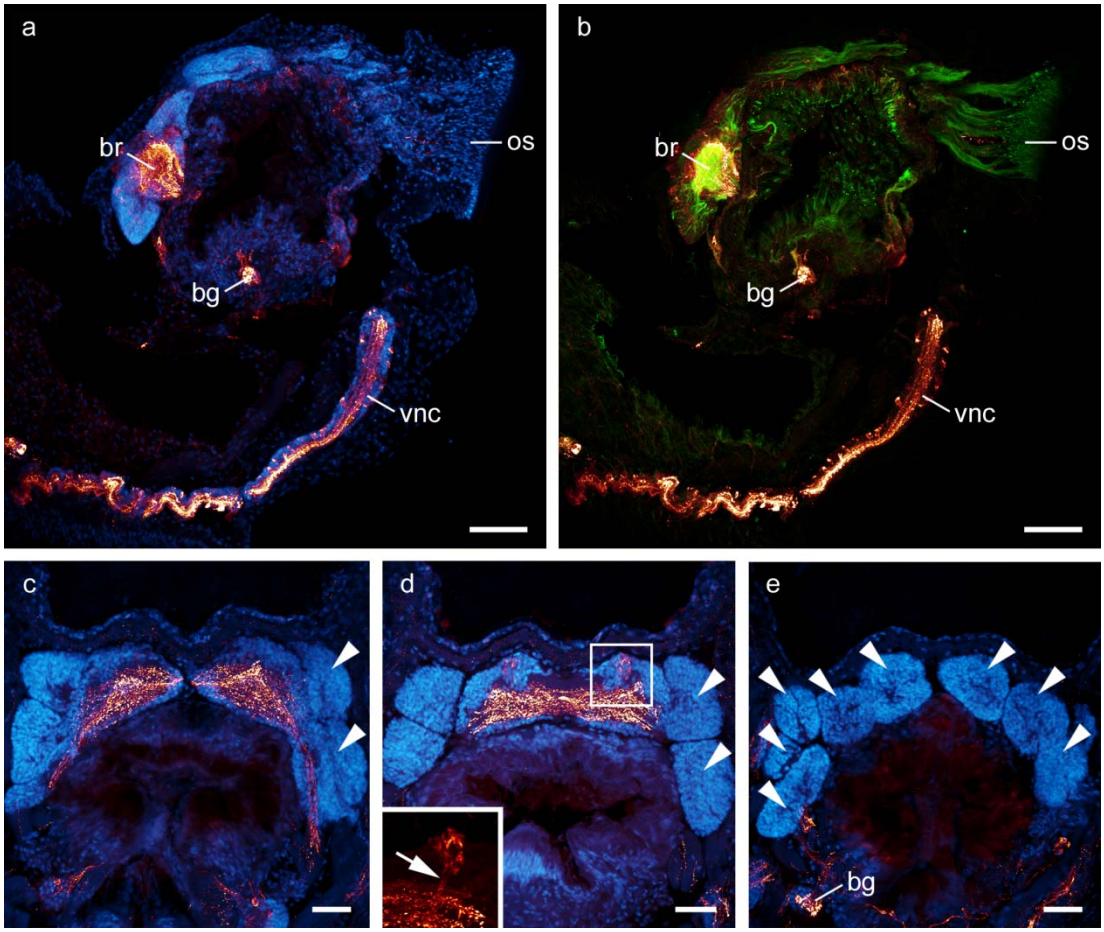

Figure 2.4 – Confocal micrograph of the nervous system of *Falcidens crossotus*. **a** Superposition image of FMRFamide-like-ir and DAPI nuclear labeling in a sagittal section showing the brain (*br*), the ventral nerve cord (*vnc*), the buccal ganglion (*bg*), and the oral shield (*os*). **b** Superposition image of anti-acetylated α-tubulin and DAPI nuclear labeling of the same section as shown in **a** demonstrating that the oral shield (*os*) is innervated by fibers that show a strong anti-acetylated α-tubulin-ir. **c-e** Superposition images of serotonin-like-ir and DAPI nuclear labeling in three consecutive horizontal sections (from ventral to dorsal) of the brain showing the number and position of the precerebral ganglia (*arrowheads*). The detail in **d** shows a cluster of serotonin-like immunoreactive somata that is connected to the central fiber mass of the brain (*arrow*). Scale bars: a,b = 80 µm; c,d,e = 40 µm.

demonstrates that the central fiber mass of the dorsal part of the brain is surrounded by a cortex of neuronal somata that is most pronounced at the posterior aspect. In the lateral area of the X-shaped neuropil the cortex forms protrusions that are notching the central fiber mass (arrow Fig. 2.5b).

In addition to the immunoreactivity in the brain, FMRFamide-like-ir was also found within the lateral, buccal, and the pedal ganglia. Comparatively weak immunoreactivity was found within the ventral and lateral nerve cords. The paired lateral ganglia are situated lateroventral to the brain to which they are connected by lateral connectives. The lateral nerve cords originate from the lateral ganglia, proceed laterally bent in a position close to the body wall and proceed longitudinally in posterior direction. Between the lateral nerve cords,

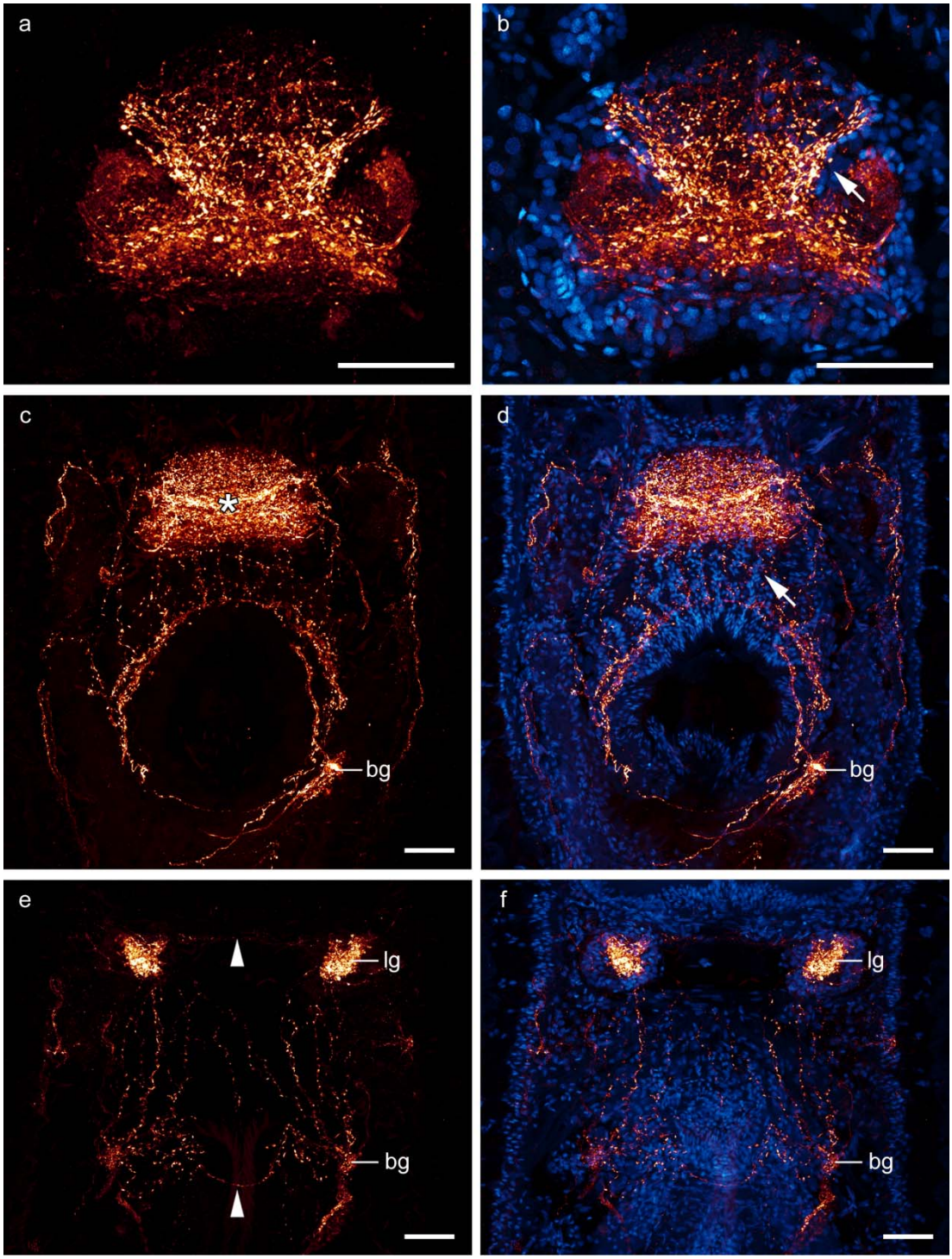

Figure 2.5 – FMRFamide-like immunoreactivity and DAPI nuclear labeling (blue) of the anterior nervous system of *Dorymenia sarsii.* Images on the left hand side (**a**,**c**,**e**) are consecutive horizontal sections from dorsal to ventral. The images on the right hand side (**b**,**d**,**f**) show the same region as superposition images of FMRFamide-like-ir and DAPI nuclear labeling. **a**,**b** Higher magnification of the dorsal part of the brain showing a band of neuronal somata lancing the central neuropil from both sides (*arrow* in **b**). **c**,**d** Ventral part of the brain showing a commissural fiber tract within the brain (*asterisk* in **c**) and posteriorly emanating fibers that are flanked by neuronal somata (*arrow* in **d**). **e**,**f** Lateral ganglia (*lg*) and ventral part of the buccal ganglia (*bg*). Both pairs of ganglia are connected by fine ventral commissures (*arrowheads* in **e**). Scale bars: a,b,c,d,e,f = 40 µm.

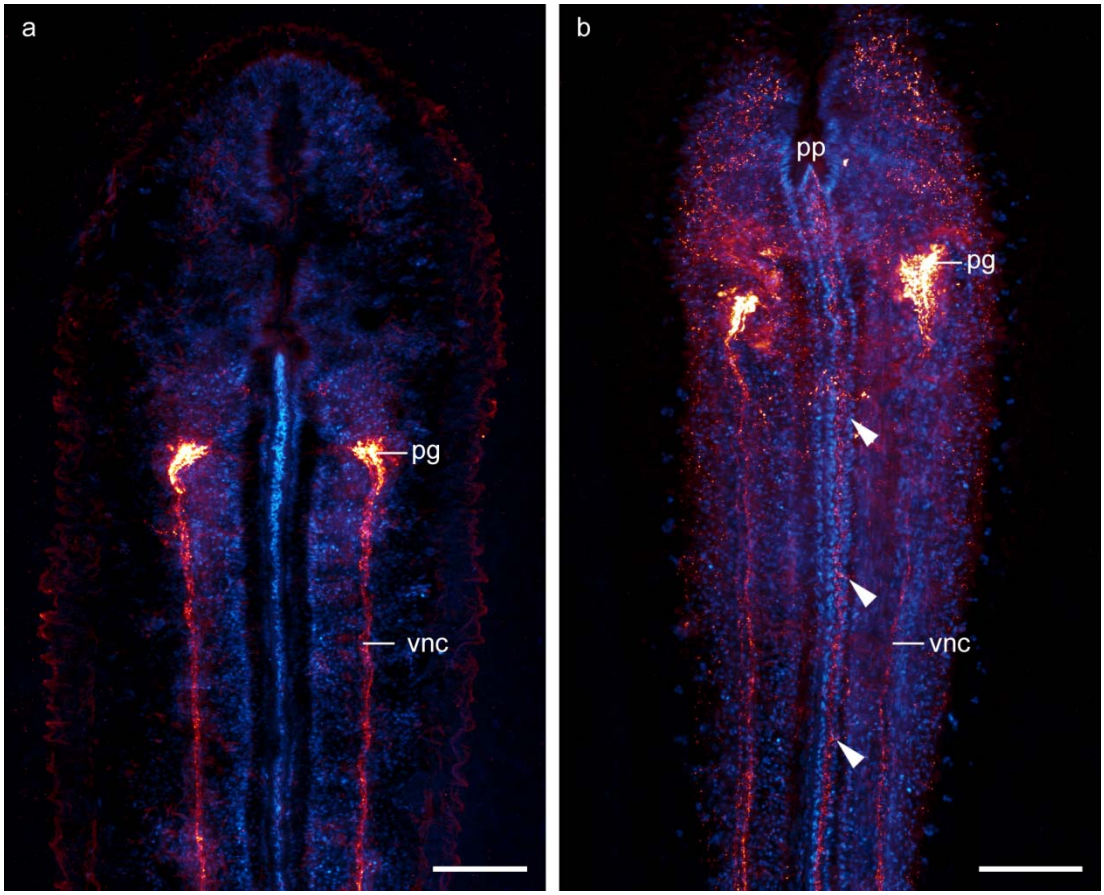

Figure 2.6 – Confocal micrograph of the anterior ventral nervous system of *Dorymenia sarsii*. a Superposition image of FMRFamide-like-ir and DAPI nuclear labeling (blue) showing the pedal ganglia (*pg*) and the paired ventral nerve cords (*vnc*). **b** Superposition image of serotonin-like-ir and DAPI nuclear labeling (blue) demonstrating an additional unpaired neurite bundle (*arrowheads*) terminating in the pedal pit (*pp*). Scale bars: a,b = 80 µm.

FMRFamide-like immunoreactive fibers emanate posteriorly from the brain (Fig. 2.5c, d) and connect it with the foregut and the paired buccal ganglia (Fig. 2.5c-f). Conspicuously, these fibers are flanked by clusters of cell nuclei (Fig. 2.5d). The buccal ganglia are interconnected by a ventral and dorsal commissure, in this way forming a ring around the radula. The pedal ganglia are situated ventral to the brain. From here, the ventral nerve cords emanate and proceed posterior (Fig. 2.6a, b). The FMRFamide- as well as the serotonin-like-ir contents appear to be very low in the ventral and lateroventral connectives. No ganglion-like swellings were observed along the lateral and ventral nerve cords.

A structure that shows pronounced serotonin-like-ir is an unpaired longitudinal neurite bundle that is situated between the paired ventral nerve cords at the midline of the animal (arrowheads Fig. 2.6b). This ventral unpaired neurite bundle is surrounded by cell nuclei and lines the ventral fold terminating in the area of the pedal pit. In contrast to the serotonin staining, FMRFamide staining does not label any unpaired longitudinal neurite bundle (Fig. 2.6a).

Polyplacophora

Lepidochitona cinerea

The central nervous system of *L. cinerea* comprises two major condensations: The cerebrobuccal ring that is situated anterior to the mouth opening and the subradular ganglion that is located posterior to the mouth opening (Fig. 2.7a). It becomes apparent that the cerebrobuccal ring is composed of different layers (Fig. 2.7b). The inner layer is composed of neuropil that is interspersed with layers of horizontal fibers. The outer layers consist of cell nuclei (Fig. 2.7b). Medially, the cell nuclei layer posterior to the cerebrobuccal ring equals the thickness of the neuropil (90 µm). In both cell nuclei layers a partition in an anterior and a posterior portion is discernable. In addition to the pronounced cell nuclei layers that surround the neuropil core of the cerebrobuccal ring, the DAPI nuclear labeling shows that there are as well some very thin layers of cell nuclei within the neuropil (Fig. 2.7b).

In its posterior part, the cerebrobuccal ring splits and give rise to the paired ventral and lateral nerve cords (Fig. 2.7a). Posterior to this split the subradular connectives branch off and connect the ventral nerve cords with the heterolaterally bipartite subradular ganglion. The subradular ganglion consists of immunoreactive fibers as well as immunoreactive somata (Fig. 2.7c). The immunoreactive somata are arranged around the central neuropil mass, especially in the posterior part of the ganglion (Fig. 2.7c).

In addition to the immunoreactivity in the cerebrobuccal ring and the subradular ganglion, FMRFamide- and serotonin-like-ir was also observed in the paired ventral and lateral nerve cords as well as in the ventral and lateroventral commissures (Fig. 2.7d). Both the ventral and the lateral nerve cords consist of a longitudinal extending central neuropil that is surrounded by neuronal somata that are as well showing immunoreactivity against FMRFamide and serotonin (Fig. 2.7d). In the DAPI nuclear labeling the nerve cords are characterized by densely packed neuronal somata surrounding the median core neuropil (Fig. 2.7e). Thus the ventral and lateral nerve cords are characterized as medullary cords. The paired ventral nerve cords are on the one hand connected to each other by the ventral commissures and on the other hand connected to the lateral nerve cords by the lateroventral commissures. Both commissure types are built by immunoreactive fiber bundles but the fiber bundles are thinner in case of the lateroventral commissures (Fig. 2.7a). In the posterior end of the animal the ventral nerve cords are connected by a commissure while the lateral nerve cords fuse in the center and form a ring in the posterior part of the animal, which, like the nerve cords, consists of an inner fiber core and outer neuronal somata (Fig. 2.7a).

Acanthochitona crinita

Similar to *L. cinerea*, the anterior nervous system of *A. crinita* consists of a cerebrobuccal ring that posteriorly splits into the paired ventral and lateral nerve cords and the subradular connectives that connect the cerebrobuccal ring with the subradular ganglion (Fig. 2.8a),

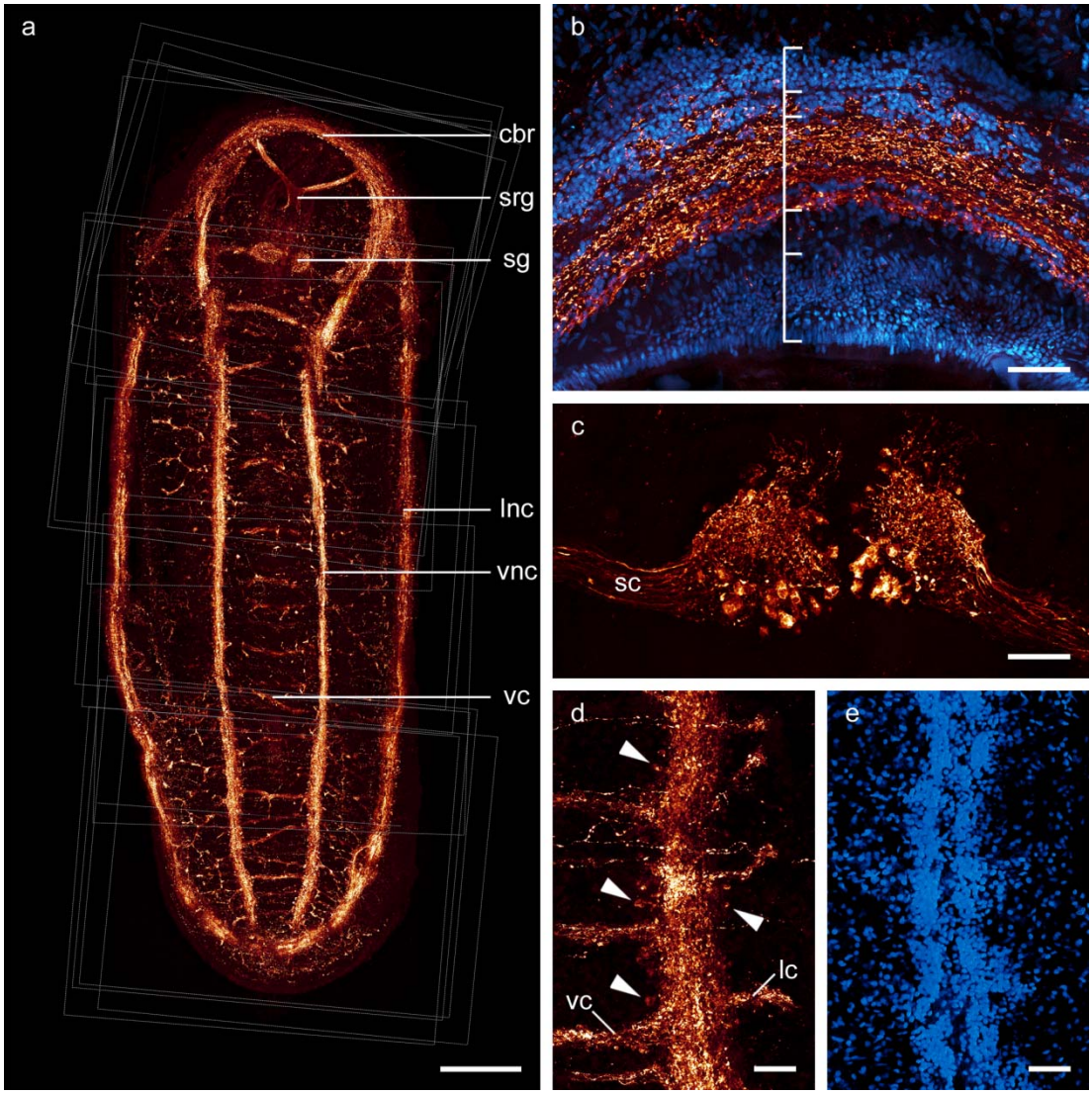

Figure 2.7 – Confocal micrograph of the nervous system of *Lepidochitona cinerea.* **a** Reconstruction composed by the overlay of 32 individual confocal images showing the central nervous system of *L. cinerea* as revealed by FMRFamide-like-ir. **b** Superposition image of serotonin-like-ir and DAPI nuclei labeling (blue) in the anterior part of the cerebrobuccal ring demonstrating that it is composed of several distinct layers. **c** Serotonin-like-ir in the subradular ganglion and subradular connectives (*sc*) illustrating that the subradular ganglion is heterolaterally bipartite. **d** Serotonin-like-ir in the ventral nerve cord, ventral commissures (*vc*), and lateroventral commissures (*lc*) showing that the ventral nerve cord is composed of immunoreactive fibers and somata (*arrowheads*). **e** DAPI nuclei labeling of the same region as shown in **d**. *cbr* cerebrobuccal ring; *lnc* lateral nerve cord; *sg* subradular ganglion; *srg* supraradular ganglion; *vc* ventral commissure; *vnc* ventral nerve cord. Scale bars: a = 400 µm; b,c,d,e = 40 µm.

but an additional supraradular ganglion could not be detected. The cerebrobuccal ring is composed of an outer layer of cell nuclei and an inner layer that consists of neuropil (Fig. 2.8b). The neuropil layer is again separated into different layers by bands of neuronal somata pervading the central fiber mass (Fig. 2.8b, c).

The ventral and lateral nerve cords are composed by an inner neuropil that is surrounded by neuronal somata.

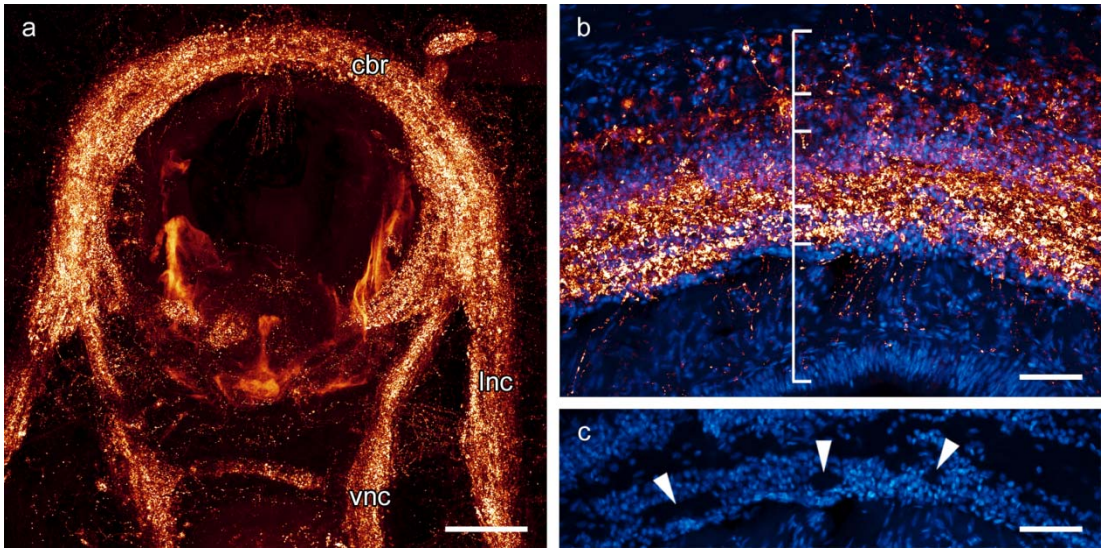

Figure 2.8 – Confocal micrograph of the nervous system of *Acanthochitona crinita*. a Reconstruction composed by the overlay of six individual confocal images showing the cerebrobuccal ring (*cbr*) and the paired ventral (*vnc*) and lateral nerve cords (*lnc*) as revealed by FMRFamide-like-ir. **b** Superposition image of FMRFamide-like-ir and DAPI nuclear labeling (blue) in the anterior part of the cerebrobuccal ring demonstrating that it is composed of several distinct layers. **c** Detail of DAPI nuclear labeling in the fourth layer (cf. **b**) revealing that the somata are not equally distributed and enclose islands of neuropil (*arrowheads*). Scale bars: a = 200 µm; b,c = 40 µm.

Scaphopoda

Antalis entalis

The central nervous system of *A. entalis* comprises three major condensations: The brain, the pleural, and the pedal ganglia. The brain is situated on the dorsal (concave) side of the animal at the base of the buccal tube. It is composed of fused ganglia that are connected by a broad commissural tract (Fig. 2.9a). FMRFamide-like-ir demonstrates that the brain consists of a central neuropil that is not subdivided into distinct compartments (Fig. 2.9a). FMRFamide-like immunoreactive somata reside laterally to the central fiber mass. The DAPI nuclear labeling demonstrates that the whole brain is covered by neuronal somata. These are most pronounced at the posterior part of the brain (Fig. 2.9a). Here, they form a pair of dense and tightly packed clusters that is clearly disconnected in the center.

The pleural ganglia are connected to the brain by short cerebropleural connectives. In contrast to the brain hemiganglia, the pleural ganglia are not fused or connected to each other (Fig. 2.9a). The pedal ganglion is situated anterio ventral to the brain, within the base of the foot. It is connected to the brain and the pleural ganglia via the fused cerebro- and pleuropedal connectives. Like the brain, the pedal ganglion consists of fused ganglia that are connected by a short commissural tract (Fig. 2.9b). Moreover the brain and the pedal ganglion are similar in size. The pleural as well as the pedal ganglion are composed of a central neuropil and some outer FMRFamide-like immunoreactive somata (Fig. 2.9a, b). The DAPI nuclear labeling demonstrates that both ganglia are evenly covered by a thin layer

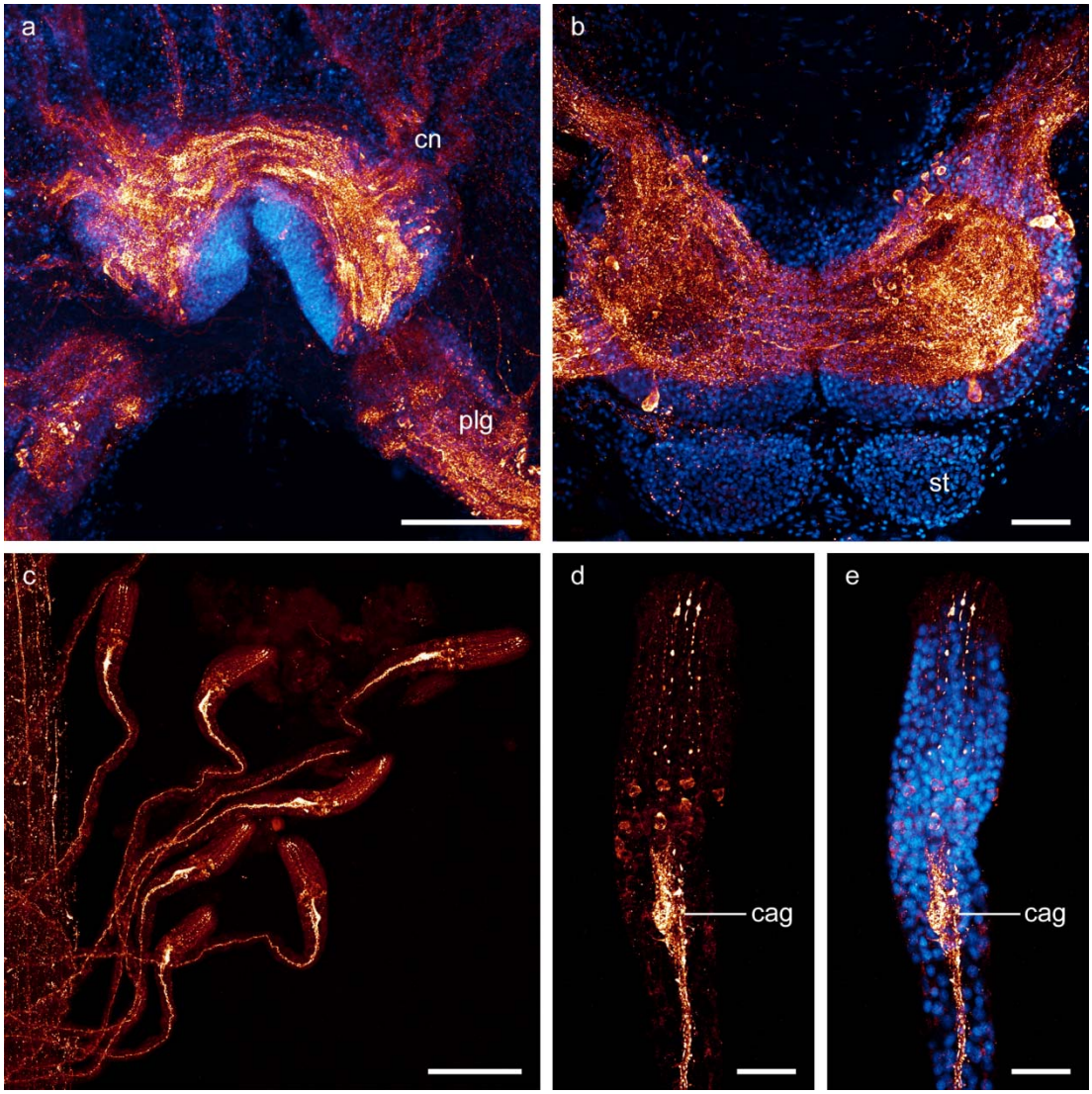

Figure 2.9 – FMRFamide-like immunoreactivity and DAPI nuclear labeling (blue) in the nervous system of *Antalis entalis.* a Superposition image of the brain and the pleural ganglia (*plg*). **b** Superposition image showing the pedal ganglion and the location of the statocysts (*st*). **c** Overview of the captacula. **d** Higher magnification of one captaculum showing the captaculum ganglion (*cag*). **e** Superposition image of the same region as shown in **d**. *cn* captacular nerves. Scale bars: a,c = 200 µm; b = 80 µm; d,e = 40 µm.

of neuronal somata, which is more pronounced in the pedal ganglion (Fig. 2.9a, b). In addition, the DAPI nuclear labeling shows the location of the paired statocysts posterior to the pedal ganglion (Fig. 2.9b).

Besides the cerebropleural and the cerebropedal connectives, the captacular nerves extend from the anterior part of the brain (Fig. 2.9a). FMRFamide-like-ir demonstrates that each captaculum is entered by a nerve and equipped with its own captacular ganglion (Fig. 2.9c-e). The nerve passes into the ganglion from which only fine nerve fibers run to the tip of the captaculum (Fig. 2.9d).

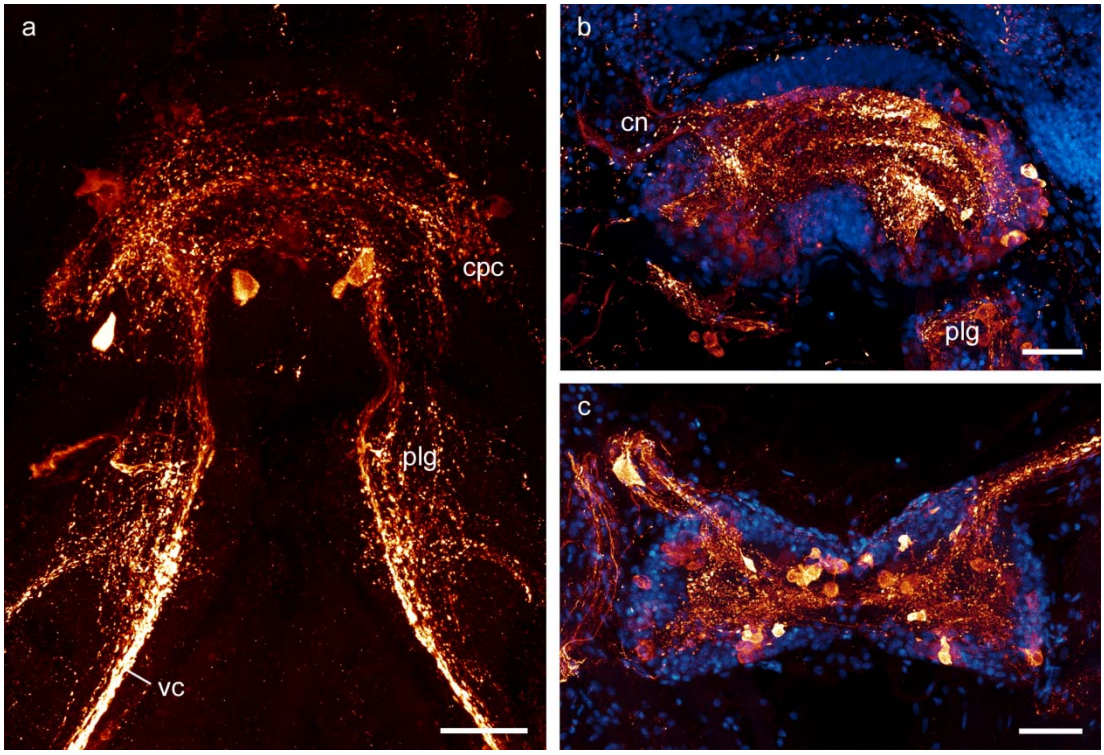

Figure 2.10 – Confocal micrograph of the nervous system of *Entalina quinquangularis*. a Overview of the brain and the pleural ganglia (*plg*) revealed by serotonin-like-ir. **b** Superposition image of FMRFamide-like-ir and DAPI nuclear labeling showing the brain, the pleural ganglia (*plg*), and the captacular nerves (*cn*). **c** Superposition image of serotonin-like-ir and DAPI nuclear labeling in the pedal ganglion. *cpc* cerebropedal connective; *vc* visceral connective. Scale bars: a,b,c = 40 µm.

Entalina quinquangularis

Serotonin- and FMRFamide-like-ir demonstrates that the nervous system of *E. quinquangularis* is composed of a brain that consists of fused ganglia and a central neuropil that is not subdivided into distinct compartments (Fig. 2.10a, b). The cerebropedal connectives emanate posterior laterally from the brain (Fig. 2.10a) and the cephalic nerves leave the brain in anterior direction (Fig. 2.10b). The pleural ganglia are situated posterior to the brain to which they are connected via short cerebropleural connectives (Fig. 2.10a). The visceral connectives emanate from the posterior part of the pleural ganglia (Fig. 2.10a). One striking difference between the brains of the two investigated scaphopod species is that there is no pronounced aggregation of DAPI labeled nuclei in the posterior part of the brain of *E. quinquangularis*. Rather, neuronal somata are distributed evenly in the anterior and the posterior part and the brain (Fig. 2.10b). The constitution of the pedal ganglion is very similar in both investigated scaphopod species but in *E. quinquangularis* the statocysts are not clearly defined in the DAPI labeling (Fig. 2.10c).

Discussion

The current study presents the first comparative immunohistochemical data on the nervous system of seven species of the lesser known mollusks caudofoveates, solenogasters, polyplacophorans, and scaphopods. Antisera against the widespread neuroactive substances FMRFamide and serotonin were used in combination with acetylated α-tubulin and the nuclear marker DAPI to label limited subset of neurons. This enables us to describe the cellular architecture of the nervous system at a fine resolution and to compare the cellular architecture of the nervous system between different species of a taxon, as well as between different class-level taxa of Mollusca.

Structure of the brain and nerve cords

Caudofoveata

The brain of both investigated caudofoveate species is divided in an anterior and a posterior section. A distinct division into anterior-posterior sections appears to be a general trait of Caudofoveata, but in contrast to that Shigeno et al. (2007) found three brain sections in *Chaetoderma japonicum*: an anterior, a posterior, and a dorsal one. The anterior lobe of *C. japonicum* is mainly characterized by the converging of the projections of the precerebral ganglia whereas the posterior lobe contains large neuropil and intense serotonin-like-ir (Shigeno et al. 2007). This constitution of the anterior and posterior lobe is also found in *Scutopus ventrolineatus* where a dense neuropil in the posterior brain section shows intense FMRFamide-like-ir. The third and dorsal lobe of *C. japonicum* is characterized by its dorsal position, small neuropils and dense serotonin-like-ir (Shigeno et al. 2007). Even though this composition is similar to the dorsal part of the brain of *S. ventrolineatus*, we do not see sufficient reason to characterize this area as a separated section of the brain.

The most remarkable structures in the nervous system of the investigated caudofoveate species are the precerebral ganglia and the ganglion-like swellings in the nerve cords. In most of the investigated *S. ventrolineatus* specimens five pairs of precerebral ganglia could clearly be identified, but also specimens with lower numbers could be observed. In *Falcidens crossotus* five pairs of precerebral ganglia could be observed. According to Salvini-Plawen (1972), the number of precerebral ganglia in caudofoveates varies between three to six pairs, with *S. ventrolineatus* possessing four pairs of precerebral ganglia and *F. crossotus* having five pairs. In contrast, a recent study by Redl and Salvini-Plawen (2009) described five pairs of precerebral ganglia in both species. Redl and Salvini-Plawen (2009) trace back the different numbers of precerebral ganglia to misinterpretations in histological sections, but according to them intraspecific variability might also be possible. The immunohistochemical results presented here also clearly argue for an intraspecific variability and against typal numbers of precerebral ganglia. In addition to the precerebral ganglia, *F. crossotus* possesses

a cluster of serotonin-like immunoreactive somata connected anteriorly to each brain hemisphere. Such a cluster was not observed in the nervous system of *S. ventrolineatus* and is also not described for *C. japonicum* (Shigeno et al. 2007) that is classified in the same family as *F. crossotus* (Chaetodermatidae). While the immunoreactivity within the precerebral ganglia is mainly restricted to neurites, this cluster exclusively consists of serotonin-like immunoreactive somata. The function of this additional nervous system concentration that is located in the same area as the precerebral ganglia remains enigmatic.

Another striking feature of the caudofoveate nervous system are the ganglion-like swellings of the ventral and lateral nerve cords. Similar serially repeated ganglion-like swellings have been described in *C. japonicum* (Shigeno et al. 2007). According to Shigeno et al. (2007), these neuronal structures do not seem to correspond with any other serial structure of the body and thus cannot be interpreted as segmentation or metamerism.

Solenogastres

In general, the results on the solenogaster nervous system presented in this study are concurrent with investigations on *Wirenia argentea* by Todt et al. (2008a). Even though the fibers in the solenogaster nervous system show FMRFamide- as well as serotonin-like-ir, the number of FMRFamide-like immunoreactive somata is low and serotonin-like immunoreactive somata are not present at all. However, in contrast to the weak labeling of ganglia in the nervous system of *W. argentea* (Todt et al. 2008a) the immunohistochemical results presented here clearly demonstrate the major ganglia of the solenogaster nervous system. This is especially true for *Dorymenia sarsii*, which was investigated by preparing sections. Merely a basal ganglion that directly rests against the brain in *W. argentea* (Todt et al. 2008a) could not be observed in the immunostaining presented here. In addition to the conspicuousness of ganglia, cerebral nerves as well as fine commissures could only hardly be observed.

The most remarkable structure in the investigated solenogaster species is the ventrally located unpaired neurite bundle that proceeds longitudinally along the ventral fold. This unpaired neurite bundle was only detected in serotonin stainings but not in FMRFamide stainings. This is especially remarkable because the serotonin levels of the remaining nervous system appear to be very low. Todt et al. (2008a) as well described serotonin-like-ir in the ventral fold of *W. argentea*, but they explained this as an unspecific binding to a substance contained in the pedal gland secretions because they did not detect any nerve bundle in a comparative position in histological or transmission electron microscopical preparations. The anti acetylated α-tubulin stainings presented here clearly demonstrate that the serotonin-like immunoreactive unpaired neurite bundle is adjacent to the pedal cilia of the ventral fold. In gastropods serotonin has been demonstrated to be involved in stimulating the beating of the pedal cilia (Audesirk et al. 1979). Therefore it is possible that the ventral unpaired neurite bundle is responsible for the coordination of ciliary movement.

Polyplacophora

The results presented here corroborate the descriptions of the polyplacophoran nervous system presented in earlier studies (Eernisse & Reynolds 1994; Moroz et al. 1994). Apart from the presence of a subradular ganglion, and the supraradular ganglion of *L. cinerea*, the nervous system lacks real ganglia. The main components of the polyplacophoran nervous system are the cerebrobuccal ring and the two pairs of nerve cords. The cerebrobuccal ring corresponds to the cerebral ganglia of other mollusks (Moroz et al. 1994). The most conspicuous feature of the cerebrobuccal ring is the division into different horizontal layers. A division of the cerebrobuccal ring was already described by Gantner (1989), who showed that the cerebrobuccal ring is divided into three different horizontal layers reflecting the lateral nerve cord, the pedal nerve cord, and the subcerebral commissure forming the cerebrobuccal ring. The arrangement of the lateral and ventral nerve cords into an inner fiber core and outer neuronal somata is also described by Moroz et al. (1994). According to Gantner (1989) the ventral and lateral nerve cords are both posteriorly connected by a commissure. However, in accordance to the neuroanatomical glossary by Richter et al. (2010) the so called suprarectal commissure turned out to be no commissure *sensu stricto* but rather a medullar connection of the lateral nerve cords.

Scaphopoda

The scaphopod nervous system is similar to the basic tetraneurous system of Conchifera with one pair of pedal and one pair of visceral nerve cords (Shimek & Steiner 1997; Reynolds & Steiner 2008). Even though the nerves extending from the ganglia were clearly visible, it was very difficult to follow them along the whole length, especially in the sections of the comparably large *Antalis entalis*.

The most striking differences between both scaphopod species investigated are the distribution of the DAPI labeled nuclei around the central neuropil of the brain and within the statocysts. While the DAPI labeled nuclei form dense and tightly packed clusters at the posterior part of the brain of *A. entalis*, such clusters are less pronounced or even not present in *Entalina quinquangularis*. The statocysts of *A. entalis* are as well clearly visible by aggregations of DAPI labeled nuclei whereas such aggregations are hardly visible posterior to the pedal ganglion in *E. quinquangularis*. These differences might reflect the evolutionary distance between those two scaphopod species. Alternatively, the significantly smaller body size of *E. quinquangularis* might be the cause. While the shell of *A. entalis* is up to 50 mm long, that of *E. quinquangularis* only comes up to one-fifth of the former.

Evolutionary considerations

Relationship among non-conchiferan molluscan taxa

Due to their worm-shape and several other presumed synapomorphies solenogasters and caudofoveates have been grouped together as Aplacophora (Scheltema 1993), but until now there is no common consent for this grouping, even though it recently has been supported by molecular phylogenetics (Kocot et al. 2011; Smith et al. 2011; Vinther et al. 2011). According to Scheltema (1993), one synapomorphy is that the tetraneural nervous system, including the cerebral commissure, the lateral and ventral nerve cords, and the suprarectal commissure, is heavily ganglionated in both solenogasters and caudofoveates, in contrast to polyplacophorans. The nerve cords of caudofoveates are indeed characterized by ganglion-like swellings, but such structures could not be detected along the nerve cords of the investigated solenogaster species where the somata are only loosely distributed. According to Todt et al. (2008a), ganglion-like swellings are present in the nerve cords of the solenogaster *Wirenia argentea*. Here, they are characterized by accumulations of somata in those areas of the nerve cords where commissures fuse with the nerve cords. Comparing the nerve cords of caudofoveates and solenogasters with that of polyplacophorans, the nerve cords of polyplacophorans clearly show no appearance of ganglion-like swellings. Rather, the polyplacophoran nerve cords are characterized by numerous and evenly distributed somata surrounding the central fiber core. In addition, the results presented here demonstrate that the lateral nerve cords of polyplacophorans are directly connected to each other posteriorly and thus a suprarectal commissure *sensu stricto* is lacking. In caudofoveates the ventral nerve cords fuse with the lateral ones in the posterior body and these fused cords are connected via a medullary cord-like commissure. In solenogasters the lateral nerve cords are posteriorly connected via an at least in several cases medullary cord-like commissure (Büchinger 1998; Todt et al. 2008a). The posterior nervous system in the three taxa is therefore similar in terms of the suprarectal “commissure” bearing scattered nuclei, but differs in other details, thus not providing support for monophyletic Aplacophora.

Comparing the structure of the brain of the three non-conchiferan molluscan taxa, it becomes apparent that exclusively the brain of caudofoveates shows a division into discrete sections along the anterior-posterior axis, a feature that is otherwise especially pronounced in gastropod and cephalopod mollusks. The brain of caudofoveates as well as the cerebrobuccal ring of polyplacophorans is composed of inner neuropil that is surrounded by a thick layer of neuronal somata. In comparison, there is only a thin layer of cell nuclei covering the neuropil in the brain of the investigated solenogaster species indicating that it is composed of less neuronal somata. These findings suggest that the brain of caudofoveates is more complex than that of solenogastres. Moreover, the brain of caudofoveates is anteriorly accompanied by precerebral ganglia. Structures similar to the precerebral ganglia of caudofoveates have as well been described for solenogasters (Salvini-Plawen 1972; Scheltema et al. 1994). These

so-called atrial ganglia are innervating the atrial sense organs of solenogastres (Büchinger 1998). In the current study, precerebral ganglia-like structures in the head of the investigated solenogaster species could not be detected. The possibility that precerebral ganglia-like structures were not labeled in the immunostainings of solenogasters has to be taken in to account, but they should have been detected at least in the DAPI nuclear labelings. Polyplacophorans show as well no evidence of precerebral ganglia-like structures.

On the basis of these results, a monophyly of Aplacophora could not be confirmed. Interestingly, the caudofoveate nervous system seems to be most highly derived within the non-conchiferan mollusks, which is remarkable considering the caudofoveates burrowing and detritus-feeding lifestyle in comparison to the predatory feeding habits of solenogasters. In annelids, in contrast, the highest cerebral complexity exists in predatory polychaetes, whereas detritus feeders have a relative simple neuroarchitecture (Heuer et al. 2010). According to the results presented here, such a correlation is clearly not applicable for non-conchiferan mollusks, strengthening the phylogenetic signal of central nervous system characters.

Position of the Scaphopoda within Conchifera

The nervous system of both proposed sister taxa to Scaphopoda, the Bivalvia (Diasoma concept) and the Cephalopoda (helcionellid concept), is highly derived, albeit in a different manner. Among others, one synapomorphy of the proposed taxon Diasoma is an epiathroid nervous system with true pedal ganglia and visceral ganglia with identical position and innervation areas (Haszprunar 1988). In an epiathroid nervous system the pleural ganglia are situated next to, or fused with, the brain, a situation that is as well present in some gastropods including most caenogastropods (Aktipis et al. 2008). Even though the central nervous system of cephalopods is well studied by immunohistochemical studies (Shigeno & Yamamoto 2002; Wollesen et al. 2008) the comparison of the anterior nervous system of scaphopods and cephalopods is complicated, because in cephalopods most of the ganglia are concentrated as lobes of a large brain. On the other hand the comparison between the scaphopod and bivalve nervous system is affected by the analogy of the scaphopod and bivalve nervous system as an adaption to their burrowing lifestyle and by the general reduction of the bivalve nervous system. Addressing the recently proposed third sister group relationship Scaphopoda – Gastropoda (Smith et al. 2011), there are distinct differences between the nervous system of scaphopods and "Archaegastropoda", the gastropods supposedly showing ancestral morphological traits. Archaegastropods have a hypoathroid nervous system (pleural ganglia close to pedal ganglia) and ventral nerve cords. Haszprunar (1988) suggests the hypoathroid state to present a gastropod apomorphy, only secondarily changed to the epiathroid state in caenogastropods. If this is the case, then the nervous system does not provide any support for a scaphopod-gastropod clade, either.

Position of Mollusca within Protostomia

When comparing the brain of mollusks with other protostome taxa it becomes apparent that the nervous system in mollusks is very variable and exhibits a wide range of differentiation. In annelids and arthropods the brain neuropil is clearly divided into several compartments (Heuer et al. 2010; Strausfeld et al. 1995). The most prominent of these neuropils are clusters of olfactory glomeruli, the paired mushroom bodies, and the unpaired central body. Although the brains of the investigated molluscan taxa are not subdivided in those different compartments, the precerebral ganglia of caudofoveates in some aspects resemble the mushroom bodies of annelids and arthropods. The mushroom bodies of annelids and arthropods perceive chemosensory information. Since the precerebral ganglia of caudofoveates are directly connected to the oral shield they are also assumed to participate in the chemosensory network (Shigeno et al. 2007). However, a clearly identifiable mushroom body *sensu stricto* that is divided into a calyx region, a peduncle, and output lobes is not present in any of the investigated molluscan species.

The most striking morphological feature of the annelid and arthropod mushroom bodies are the so-called globuli cells. Those cells are mainly characterized by their small size and densely package (Richter et al. 2010). The DAPI labeled nuclei building the precerebral ganglia of the investigated caudofoveate species are also characterized by their comparatively dense package. Those findings are in contrast to the descriptions of Shigeno et al. (2007) for *Chaetoderma japonicum*. Looking at the other molluscan taxa investigated, the solenogaster and polyplacophoran species do not possess clusters of small diameter cells that would be comparable to the globuli cells of arthropods and annelids. In contrast, clusters of densely packed DAPI labeled nuclei are present in the posterior part of the brain of the scaphopod *Antalis entalis*. However, globuli cells have not been described for the complex brain of cephalopods (Wollesen et al. 2008), a potential sister group of scaphopods. Looking at the remaining taxa of conchiferan mollusks, globuli-like cells are only present within the procerebrum of pulmonate gastropods (Ratté & Chase 1997), a group of highly derived snails with special adaptations to limnic or terrestrial habitats. Like the mushroom bodies of annelids and arthropods and the precerebral ganglia of caudofoveate mollusks the procerebrum of gastropods serves within the olfactory pathway (Ratté & Chase 1997). Considering the absence of globuli cell clusters in the brain of most of the molluscan taxa and excluding the possibility of repeated reduction, a homology of the molluscan globuli cells to that of arthropods and annelids is unlikely. Molecular fingerprint studies that have already been carried out for the mushroom bodies of annelids and arthropods (Tomer et al. 2010) can help to elucidate whether centers homologous to mushroom bodies are present outside of Annelida and Arthropoda.

In summary, the results presented here demonstrate the great variability of the molluscan brain and a high plasticity at least in part in response to lifestyle. The profound structural differences in the nervous system of caudofoveates, solenogastres,

polyplacophorans, and conchiferans – herein exemplified by the scaphopods – suggest a deep time origin of morphological diversification within the Mollusca.

3 Neuroanatomy of Nemertea

Introduction

Nemertea is an undoubtedly monophyletic group of vermiform unsegmented spiralians. Most species are marine, inhabiting a wide range of interstitial, benthic, or pelagic habitats. There are some representatives that have invaded limnic or moist terrestrial environments. To date, about 1280 species have been described (Kajihara et al. 2008). Nemerteans possess an eversible proboscis, to catch and intoxicate their prey organisms. Most benthic nemerteans hunt actively at night at low tide pursuing their prey animals by following them in their tracks (Amerongen & Chia 1982; Thiel 1998; Thiel & Kruse 2001). For this purpose they use a number of different sensory organs that are mainly situated in the frontal region of the animals (Gibson 1982). The most conspicuous sensory organs are the cerebral organs. These spherical structures are closely associated with the brain and have been demonstrated to play a role in chemoreception (Ling 1969; Amerongen & Chia 1982).

Descriptions of the gross anatomy of the central nervous system of nemerteans were first made in the late 19th and early 20th century. According to these authors, the central nervous system of nemerteans consists basically of a pair of cerebral ganglia and a pair of lateral nerve cords. The cerebral ganglia are arranged as dorsal and ventral lobes. The two dorsal as well as the two ventral lobes are mutually interconnected by a dorsal and a ventral commissure, respectively (Bürger 1895; McIntosh 1900; Gibson 1972). The cerebral ganglia thus enclose the anterior portion of the rhynchocoel.

Due to morphological characters like the acoelomate body organization, the architecture of the nervous system, the sense organs, and the protonephridial excretory structures, Nemertea were traditionally placed close to Platyhelminthes (Nielsen 2001). In contrast, the fate of the trochoblast cells gives some evidence for including nemerteans into Trochozoa (Maslakova et al. 2004). Moreover, recent molecular studies have produced ambiguous results. Even though none of the molecular based studies found support for a relationship between Nemertea and Platyhelminthes, the placement of Nemertea within Lophotrochozoa varies between different studies (Turbeville & Smith 2007; Dunn et al. 2008; Struck & Fisse 2008; Hejnol et al. 2009; Paps et al. 2009; Podsiadlowski et al. 2009). Therefore, additional data are necessary to unravel the phylogenetic position of nemerteans.

Searching for novel characters, one promising structure is the nervous system. The methodological backbone of a discipline, that is now being termed "neurophylogeny", has been outlined in a number of publications (Kutsch & Breidbach 1994; Harzsch 2006). In the last decade neuroanatomical characters have already been used successfully for the inference of phylogenetic relationships within the arthropods (Harzsch & Waloszek 2000; Strausfeld et al. 2006a). Recently, the neuroanatomy of various lophotrochozoan taxa has been studied using immunohistochemical methods (Müller 2006; Shigeno et al. 2007; Heuer & Loesel 2008a; Kristof et al. 2008; Todt et al. 2008a; Wollesen et al. 2008; Heuer et al. 2010). Even though immunohistochemical investigations of the larval nervous system of nemerteans have been published (Hay-Schmidt 1990; Chernyshev & Magarlamov 2010; Maslakova 2010), actually no data are available for adult nemerteans.

In the present study, antibodies directed against FMRFamide and serotonin were used to reveal the structure of the central and peripheral nervous system of the nemertean *Lineus viridis*. These two antisera are known to label subsets of neurons in all major animal clades and are frequently used in neuroanatomical studies across the animal kingdom. Therefore, these markers facilitate the comparison of nemerteans to other taxa. Since one aim of this study was to describe the nervous system of a representative of nemerteans in detail, additional DAPI nuclear labelings were done to obtain a complete view of the nervous system.

Materials and Methods

Specimens of *Lineus viridis* (Müller, 1774) (Nemertea, Anopla, Heteronemertea) were collected during field trips on the Isle of Sylt (Germany) during day low tide. Animals were found under stones or shells of *Crassostrea gigas*. To reveal details of the neuroarchitecture of *L. viridis*, ten specimens were analyzed by a combination of immunohistochemistry and DAPI nuclear labeling.

In principle, immunohistochemistry was performed as described in Heuer and Loesel (2008a) and Heuer et al. (2010). Animals were anesthetized with a 7% $MgCl_2$ solution in seawater. The worms were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS) at 4°C. After fixation all animals were decapitated and the head regions were washed several times in PBS. For vibratome-sectioning (VT1000S, Leica Microsystems, Wetzlar, Germany) the head regions were embedded in a gelatine/albumin medium. After hardening the gelatine/albumin blocks overnight in 14% Formalin in PBS at 4°C, they were cut into sections of 80 µm in thickness. The sections were then washed in PBS with 0.1% Triton X-100 (TX) and pre-incubated overnight in a blocking solution of PBS containing 0.5% TX and 5% normal swine serum (Jackson ImmunoResearch, West Grove, PA). Primary antibodies were added directly to the blocking solution and incubated

overnight at room temperature. The primary antibodies anti-FMRFamide (ImmunoStar, Hudson, WI) and anti-serotonin (Sigma-Aldrich, Saint Louis, MO) were both used at a dilution of 1:20000. After incubation with primary antibodies the sections were again washed in PBS with 0.1% TX and were then incubated overnight with the secondary antibody conjugated to fluorophore (Cy3-conjucated goat anti-rabbit; Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:2000 in PBS containing 0.5% TX and 1% normal swine serum. Following removal of the secondary antiserum the sections were incubated with the nuclear marker DAPI (4',6-Diamidino-2-phenylindole, dilactate; Sigma-Aldrich, Steinheim, Germany) at a dilution of 1:1000 in PBS for 10 min. Subsequently, sections were rinsed again in several changes of PBS containing 0.1% TX and then mounted on chrome alum/gelatine-coated glass slides under glass coverslips using Elvanol (mounting medium for fluorescent staining after Rodriguez and Deinhardt (1960)). Preparations were analyzed with a confocal laser scanning microscope (TCS SP2, Leica Microsystems, Wetzlar, Germany). A helium/neon laser (excitation wavelength 543 nm, detection range 555-700 nm) was used to detect Cy3 fluorescence, DAPI fluorescence was detected with a diode laser (excitation wavelength 405 nm, detection range 410-550 nm). The resulting image stacks were collapsed using the "maximal projection" tool of the TCS SP2 Leica confocal software. These images were finally processed using global imaging enhancement procedures (contrast, brightness) and superposition functions of Adobe Photoshop CS.

The nomenclature used for describing neuroanatomical structures in this study conforms to Richter et al. (2010).

Results

The brain of *Lineus viridis* is located inside the head of the animal. It measures approximately half of the head's width. In the living animal the brain can be identified as a reddish structure in the shape of an inverted U that shines through the semitransparent tissue of the body wall. It is situated just anterior to the mouth opening. The brain consists of a dorsal and a ventral part (Fig. 3.1a, b). Both parts are clearly separated into two lobes that are connected by a dorsal and a ventral commissural tract (Fig. 1.4b, 3.1c). Prominent sensory structures in the head of *L. viridis* are the cerebral organs that are connected posteriorly to the dorsal lobes of the brain and a number of eyes that are arranged along the lateral margin of the animal's head. The ventral lobes of the brain merge with the ventral nerve cords.

Brain

All parts of the brain are composed of neuropil that is surrounded by a massive layer of densely packed neuronal somata (Fig. 3.1a-c). A partition of the brain neuropil was not

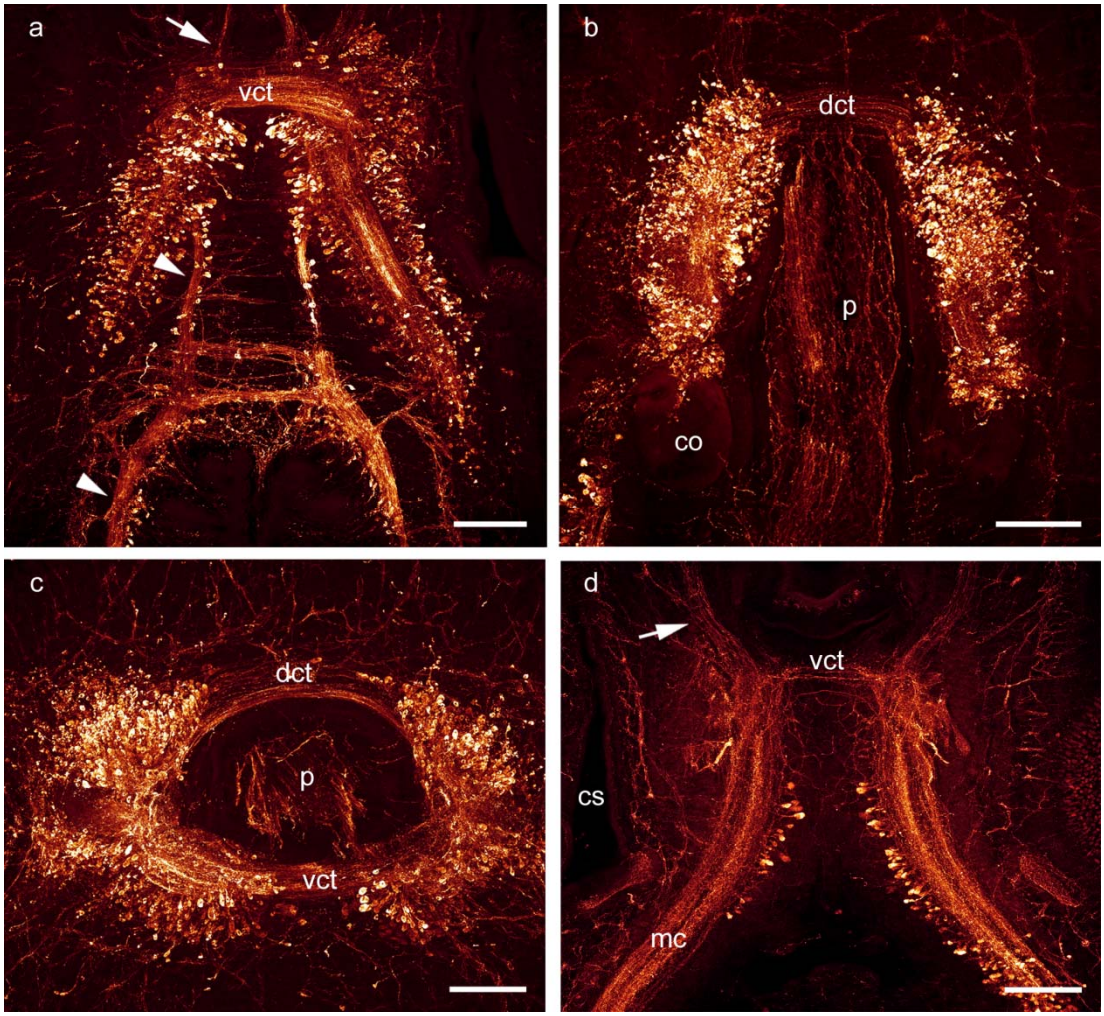

Figure 3.1 - Neuroarchitecture of the brain of *Lineus viridis*. a FMRFamide-like-ir in a horizontal section through the ventral lobes of the brain. The brain is composed of neuropil that is surrounded by densely packed somata. The ventral lobes are connected by the ventral commissural tract (*vct*). Medially, the esophageal nerves (*arrowheads*) arise from the ventral lobes. Cephalic nerves (*arrow*) extend from the ventral lobes of the brain towards the anterior tip of the animal. **b** FMRFamide-like-ir in a horizontal section through the dorsal lobes of the brain. The dorsal lobes are connected by a dorsal commissural tract (*dct*), as well. The brain is penetrated by the proboscis (*p*) which exhibits a cylindrical plexus in the immunostainings. The cerebral organs (*co*) are attached to the brain posteriorly. **c** FMRFamide-like-ir in a cross sections demonstrates that the brain and its commissural tracts form a ring surrounding the proboscis (*p*). **d** Serotonin-like-ir in a horizontal section through the most ventral part of the ventral lobe. Posteriorly, the ventral part of the ventral lobes merges with the paired medullary cords (*mc*). Laterally, the cephalic slits (*cs*) proceeds longitudinally on each side of the head. Scale bars: a,b,c,d = 200 μm.

discernable in the branching pattern of immunolabeled neurons. On each side, the anterior part of each dorsal lobe is connected with the anterior part of the ipsilateral ventral lobe. Both ventral and dorsal lobes are heterolaterally connected by an anterior commissural tract. The more prominent ventral commissural tract is situated anterior to the dorsal one and proceeds below the rhynchocoel, while the dorsal commissural tract connects the two lobes above the rhynchocoel. Accordingly, the frontal part of the brain forms a ring that surrounds

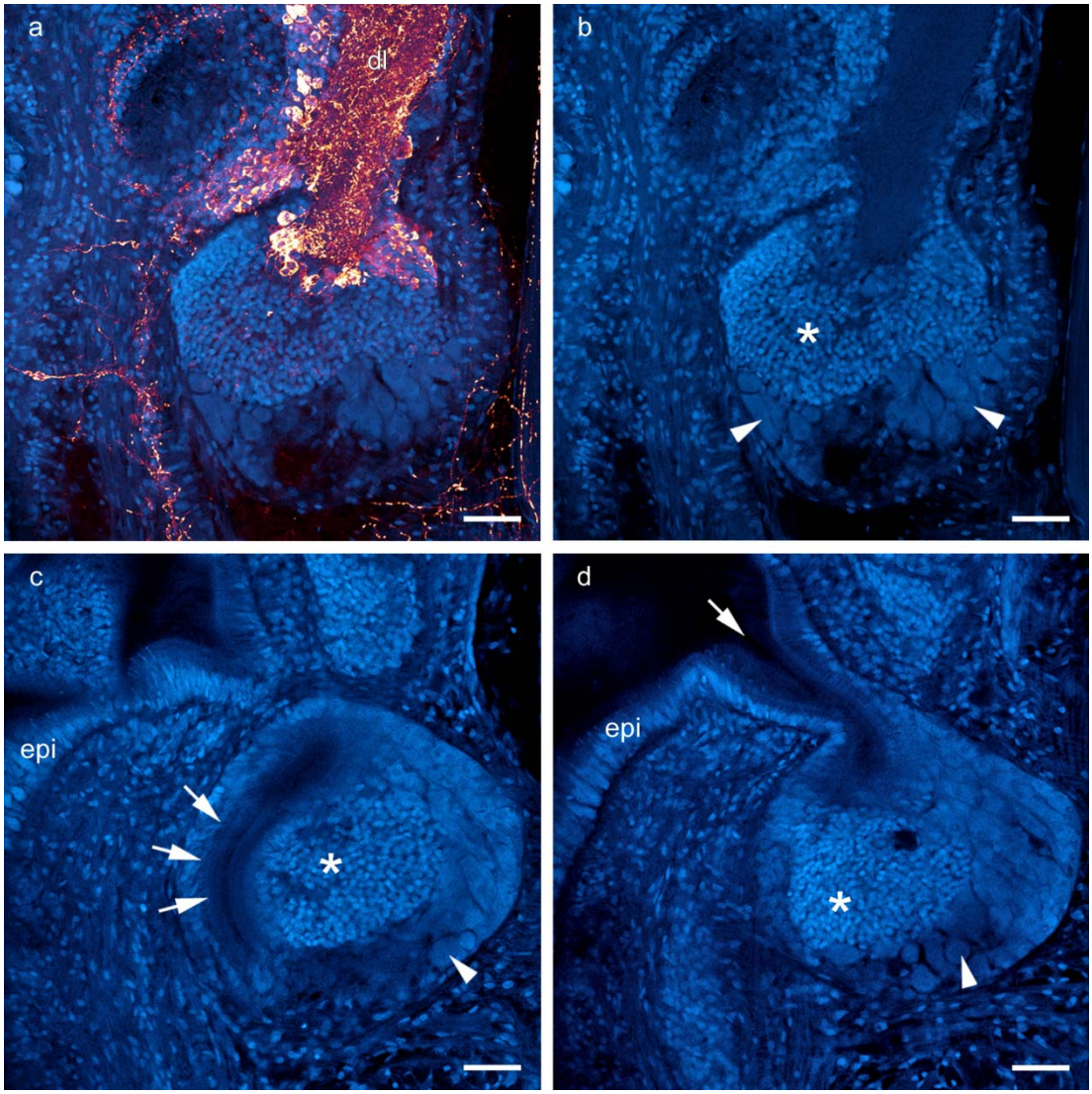

Figure 3.2 - Morphology of the cerebral organ of *Lineus viridis*. a Overlay image of DAPI nuclear labeling in blue and FMRFamide-like immunostaining in red. The cerebral organ is attached to the dorsal lobe (*dl*) of the brain. Its anterior part is innervated by neurites originating from the brain. **b** The remainder of the cerebral organ is filled with densely packed small diameter somata (*asterisk*) and large diameter cells that contain voluminous cell nuclei (*arrowheads*). **c** A canal (*arrows*) runs along the lateral aspect of the cerebral organ. **d** In the most ventral part of the cerebral organ this canal opens into a ciliated epidermal canal (*arrow*) connecting the cerebral organ with the environment. *epi* epidermis. Scale bars: a,b,c,d = 40 µm.

the rhynchocoel and the proboscis (Fig. 3.1.c). From here the four individual lobes extend posteriorly. The dorsal lobes bifurcate to form an inferior and superior branch while the ventral lobes merge with the paired medullary cords (Fig. 3.1d). Cephalic nerves extend from the ventral and dorsal lobes of the brain towards the anterior tip of the animal (Fig. 3.1a, d). The paired esophageal nerves emanate posteriorly from the ventral lobes of the brain and are interconnected by the esophageal commissures (Fig. 3.1a). Each esophageal nerve ramifies at the level of the mouth opening. The paired proboscis nerves originate from the ventral commissural tract.

Cerebral organ

Like in all lineids, in *L. viridis* a pair of cerebral organs is posteriorly attached to the dorsal lobes of the brain (Fig. 3.1b, 3.2a). While the superior branch of the dorsal lobe rests on the cerebral organ, the inferior branch deeply extends into it. Thus each cerebral organ is innervated by immunoreactive neurites originating in the brain (Fig. 3.2a). The neurites are surrounded by a cluster of densely packed, small diameter somata (Fig. 3.2a, b). More peripherally, these somata are covered by several layers of cells that are larger in diameter and contain voluminous cell nuclei (Fig. 3.2b-d). Here, the cephalic organ is in close contact to blood vessels. A duct runs along the lateral aspect of the cerebral organ (Fig. 3.2c) and opens ventrally into an epidermal ciliated canal (Fig. 3.2d). The canal of each cerebral organ widens to a cephalic slit that proceeds longitudinally on each side of the head and connects the cerebral organ with the environment.

Nerve cords and peripheral nervous system

The lateral nerve cords originate in the ventral lobes, extend posteriorly, and are embedded between the inner circular muscle layer and the outer longitudinal muscle layer. Serotonin-like immunoreactivity demonstrates that the nerve cord is not only built by immunoreactive neurites. Rather are immunoreactive neurites accompanied by immunoreactive somata that are arranged in a U-shaped manner around the neurites (Fig. 3.3a, b) and characterize the lateral nerve cord of *L. viridis* as a medullary cord. As in the brain, the neurites are separated from the somata by an inner neurilemma and are enclosed by an outer neurilemma. The paired medullary cords are mutually interconnected by circular neurite bundles of the commissural plexus (Fig. 3.3b). The medullary cords as well as the commissural plexus are located between a pronounced subepidermal plexus and a stomatogastric plexus (Fig. 3.3a, b). Numerous interconnections exist between the commissural plexus and both the subepidermal and the stomatogastric plexus. An additional immunoreactive plexus is located in the proboscis wall (Fig. 3.3b). The different plexus are not only characterized by their location within the animal but differ in their arrangement of individual neurites. Neurites of the subepidermal plexus are arranged diffuse and netlike (Fig. 3.3c) while in the commissural plexus the main neurite bundles are predominantly arranged in a highly regular circular fashion (Fig. 3.3d).

Discussion

In this study, the neuroanatomy of the nervous system of *Lineus viridis* was revealed utilizing antibodies against FMRFamide and serotonin. In addition, DAPI nuclear labeling

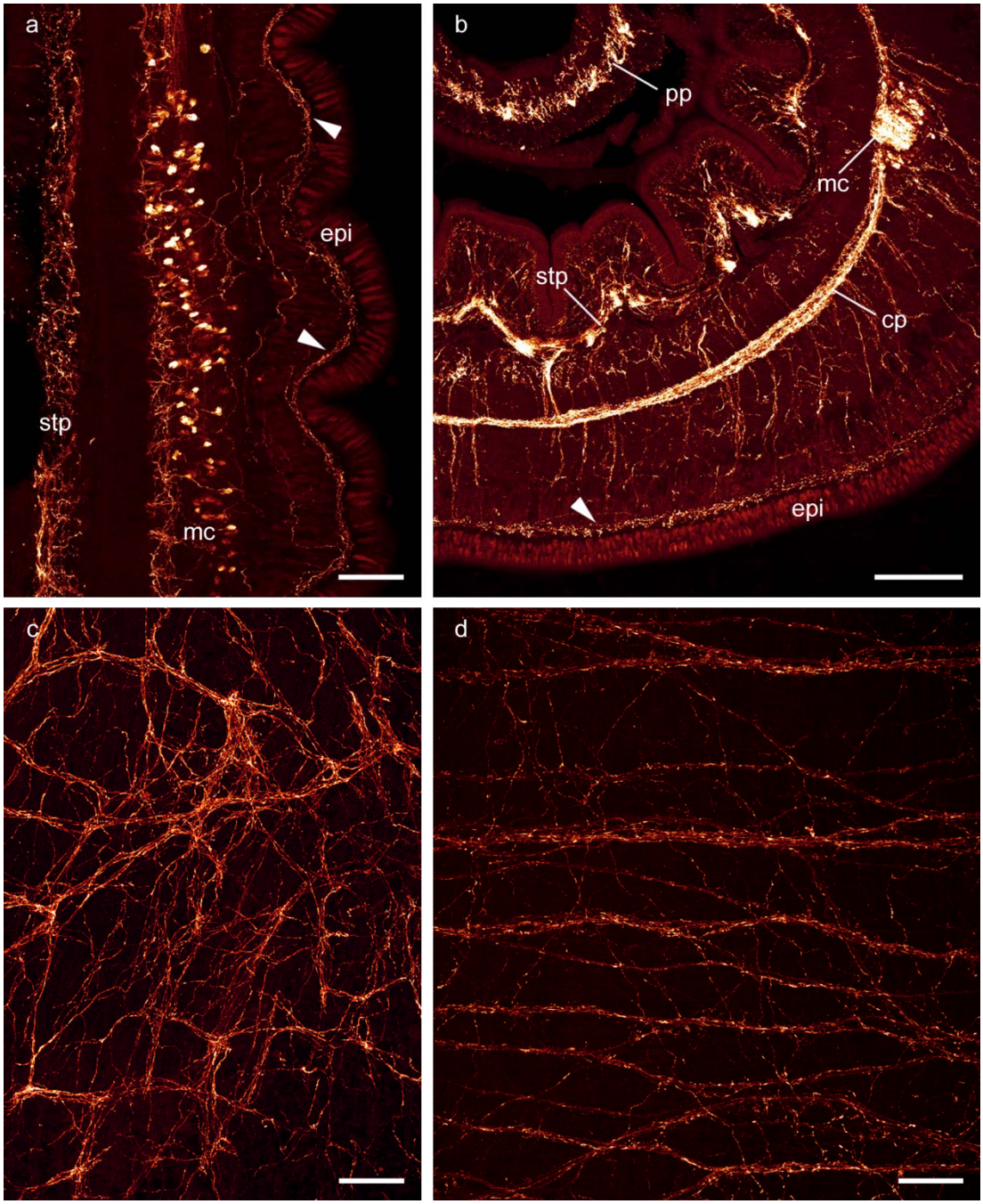

Figure 3.3 - General anatomy of the peripheral nervous system of *Lineus viridis*. **a**: Horizontal section of the post-cerebral nervous system showing serotonin-like immunoreactive neurites and somata of the medullary cord (*mc*) as well as the subepidermal plexus (*arrowheads*) and the stomatogastric plexus (*stp*). **b**: Transverse section showing FMRFamide-like-ir in the medullary cord (*mc*), the subepidermal plexus (*arrowhead*), the commissural plexus (*cp*), the stomatogastric plexus (*stp*), and the plexus of the proboscis wall (*pp*). **c**: Serotonin-like-ir in a horizontal section reveals a diffuse arrangement of neurites in the subepidermal plexus (corresponding to *arrowheads* in **a** and **b**). **d**: FMRFamide-like-ir in a horizontal section demonstrates that neurites of the commissural plexus are preferentially oriented in circular manner. *epi* epidermis. Scale bars: a,c,d = 80 µm; b = 200 µm.

were applied to visualize the gross anatomy of the entire nervous system. Applying a combination of different staining methods is a useful approach when trying to describe the neuroanatomy of a species under investigation in as much detail as possible, since each given method reveals only a limited number of aspects of the nervous system. For example, immunohistochemistry marks only a specific subset of neurons in the nervous system and is therefore useful to describe the cellular architecture of the nervous system at a fine resolution.

Peripheral nervous system

In the peripheral nervous system of *L. viridis* four distinct nerve plexus are present (Fig. 3.3): The subepidermal plexus, the commissural plexus, the stomatogastric plexus, and the proboscidial plexus. The neurites of the subepidermal plexus are arranged in a diffuse net-like manner. In contrast, the neurites of the commissural plexus are arranged in a more regular way. Turbeville and Ruppert (1985) described subepidermal and proboscidal nerve plexus in palaeonemertean species. But these authors did not provide any information on the arrangement of the neurites in the plexus. Nevertheless, the available data suggests that a proboscidial and a subepidermal plexus are present in the ground pattern of the nemerteans. Comparing the peripheral nervous system of *L. viridis* with that of non nemerteans it is noticeable that subepidermal plexus have also been reported in platyhelminthes (Baguñà & Ballester 1978; Reuter & Gustafsson 1995) and several lophotrochozoan clades such as mollusks (Moroz et al. 1994), annelids (Orrhage & Müller 2005; Müller 2006), and phoronids (Herrmann 1997), albeit they have not been analyzed in detail in these phyla. In ecdysozoans or deuterostomes subepidermal plexus have not been described so far (Schmidt-Rhaesa 2007).

Central nervous system

The central nervous system of *L. viridis* consists primarily of the brain and a pair of lateral nerve cords of the medullary type (somata distributed evenly throughout the entire length). Medullary cords are also described for platyhelminthes (Reuter & Gustafsson 1995), basal mollusks (Eernisse & Reynolds 1994; Moroz et al. 1994; Scheltema et al. 1994; Todt et al. 2008a), as well as for some polychaetes (Golding 1992; Orrhage & Müller 2005). At current state of knowledge, it is not possible to homologize the medullary cords of these different spiralian taxa.

The brain of *L. viridis* is subdivided into four lobes. Each lobe is composed of a central neuropil that is surrounded by a massive layer of neuronal somata. Bürger (1895) and Bianchi (1969) classified four different types of somata in the brain of the heteronemertean *Cerebratulus marginatus*. In contrast, in palaeonemerteans only one or two types of neuronal

somata can be discriminated (Bürger 1895), reflecting the complexity of the heteronemertean brain. The heteronemertean *L. viridis* is a foraging hunter (Nordhausen 1988) and has an elaborate mating behavior (Bartolomaeus 1984) which may explain the well developed nervous and sensory system. Moreover, lineid species like *L. viridis* show a higher complexity of the brain than other heteronemerteans (Bürger 1895; Gibson 1982). This may be related to the characteristics of the cerebral organ. Although equally termed sensory organs are also described in hoplonemerteans and tubulanid palaeonemerteans these differ in structure and position. The simplest condition of cerebral organs is found in some palaeonemerteans where they comprise simple lateral sensory pits. In other palaeonemerteans a short duct leads directly to a subepidermal chamber (Gibson 1972). According to Gibson (1972), further development is achieved by elongation and curving of the duct (cerebral canal). Some palaeo- and the hoplonemerteans have an inner sensory portion of the cerebral canal embedded in a complex of nervous and glandular cells that is connected to the brain (Gibson 1972). Only in lineid species the cerebral organs correlate with a division of the dorsal lobe of the brain into a blind ending superior branch and an inferior branch that is connected to the cerebral organ. The cerebral organs of *L. viridis* have intimate contact to the environment and are directly innervated by neurites of the brain (Fig. 3.2a). It is very likely that these structures serve as chemo-sensitive organs and allow the animal to orientate in its environment (Ferraris 1985). The structure of the cells in the periphery of the cerebral organ (Fig. 3.2b-d) suggests that these cells presumably are glandular cells. Together with the proximity of the cerebral organ to the blood vessel this is considered an evidence for its neurosecretory (neuroendocrine) function (Scharrer 1941; Ling 1969; Ling 1970; Ferraris 1985).

The brain neuropil of *L. viridis* shows no partition of immunostained fibers. In this respect, nemertean brain architecture differs from that of other protostome clades with elaborate brains such as vagile annelids and arthropods. Whereas the pattern of immunostaining in the brain of *L. viridis* appears to be evenly distributed with no obvious boundaries, in annelids and arthropods the brain neuropil is clearly divided into several subcompartments (Strausfeld et al. 1995) that are again termed neuropils. The most prominent of these neuropils in annelids as well as in arthropods are clusters of olfactory glomeruli, the elaborate paired mushroom bodies, and – in arthropods - the unpaired central body whose phylogenetic affinities to the unpaired midline neuropil occasionally described in annelids remains a matter of debate (Loesel 2004; Heuer & Loesel 2009; Heuer et al. 2010; Loesel & Heuer 2010).

Although the brain of *L. viridis* is not subdivided in those different neuropils, there is one structure that resembles the mushroom bodies of annelids and arthropods. The centre of the cerebral organ is formed by a cluster of densely packed small diameter somata which resemble the globuli cells that form the annelid and arthropod mushroom bodies. In the latter two groups the mushroom bodies perceive chemosensory information which is also assumed

for the cerebral organs of nemerteans. However, even though the cerebral organs are innervated by immunoreactive neurites, the immunostainings do not reveal a neuropil that is similar to the typical mushroom body peduncle. The structures of the cerebral organ, which implicate a neurosecretory function, argue as well against the notion that the cerebral organs might represent modified mushroom bodies. Assuming that the cerebral organ does not represent a modified mushroom body, alternative explanations are conceivable. Another brain associated organ with a comparable location is the nuchal organ of polychaete annelids which shares certain similarities with the nemertean cerebral organ. Nuchal organs presumably play a role in chemosensation, as well. They usually occur pair wise and are innervated directly from the brain (Purschke 1997). Based on the current morphological data we can neither postulate homology of the cerebral organ to mushroom bodies or to nuchal organs nor exclude the possibility of an independent origin of these structures.

In search for the urbilaterian brain

Recent molecular fingerprint data indicate that the origin of higher brain centers (e.g. the mushroom bodies) date back to the last common ancestor of all bilaterians and should therefore be present in all bilaterian clades (Tomer et al. 2010). For example, molecular fingerprint data provide strong evidence for a homology of insect and annelid mushroom bodies (Tomer et al. 2010). This notion is supported by morphological data which demonstrate that the neuroarchitecture of the mushroom bodies of annelids is in many details identical to that of arthropods (Heuer & Loesel 2008a; Heuer & Loesel 2008b; Loesel & Heuer 2010). In addition, the presence of mushroom bodies has been demonstrated for a wide variety of errant polychaetes (Heuer et al. 2010). In contrast, the complex brain of cephalopod mollusks does not reveal such structures (Wollesen et al. 2008). In the present study, we were also not able to clearly demonstrate the presence of higher brain centers like the mushroom bodies in *L. viridis*. Classical studies into the organization of the nervous system of nemerteans (Bürger 1895; McIntosh 1900) have never revealed any structure resembling mushroom bodies of annelids or arthropods. Provided that these structures belong to a hypothesized urbilaterian brain, additional immunohistochemical studies in different nemertean species may uncover mushroom bodies or their homologous structures. However, one should consider the possible homology of certain cell clusters in the nemertean brain without having the typical mushroom like structure. In the end, molecular fingerprint studies (Tomer et al. 2010) would be necessary to draw a final conclusion whether centers homologous to mushroom bodies are present outside of Annelida and Arthropoda.

4 General Discussion

As a result from the new animal phylogeny and the nevertheless disputed grouping of major animal clades, the field of comparative neuroanatomy emerged as a discipline that can be helpful for the inference of phylogenetic relationships. However, until the beginning of the *Deep Metazoan Phylogeny* program detailed descriptions of the central nervous system of numerous protostome clades were lacking. For this reason, the main objective of the present thesis was to add new neuroanatomical data for so far poorly investigated taxa.

The first part of the thesis focused on the lesser-known branches of the phylum Mollusca: Solenogastres, Caudofoveata, Polyplacophora, and Scaphopoda. In comparing the neuroanatomy of those different taxa, this study has demonstrated that the brain of caudofoveates is most complex among the non-conchiferan taxa because it exclusively shows a division into discrete sections. The neuroanatomical data presented here, are therefore not in accordance with the Adenopoda hypothesis placing Caudofoveata basal within Mollusca. In addition, the finding that the brain of caudofoveates is most complex within non-conchiferan mollusks is remarkable considering the caudofoveate burrowing and detritus feeding lifestyle. Taking the results of both aplacophoran taxa together, the brain as well as the nerve cords of Caudofoveata and Solenogastres are built in a conspicuous different manner. Moreover, structures that are found in the nervous system of both aplacophoran taxa are as well present in the third non-conchiferan group, the Polyplacophora. Thus a monophyly of Aplacophora could not be confirmed. However, due to the great diversity of the phylum Mollusca resulting as well in a great variability of the nervous system, conclusions about the phylogenetic relationship among different molluscan taxa could not be drawn.

The second part of the present thesis concentrated on the neuroanatomy of one representative of the phylum Nemertea for which up to the present study the only investigation of the nervous system was from the late 19th to the early 20th century. The results presented here overall corroborate the observations of these earlier authors, but offers more details on the internal composition of the brain and the cerebral organ. In addition, by presenting also a detailed view on the peripheral nervous system, this study reveals that nemerteans possess a pronounced subepidermal plexus, indicating that a subepidermal plexus most likely belongs to the ground pattern of Lophotrochozoa.

Evolution of higher brain centers

In general, there are two hypothesis of central nervous system evolution (Hirth & Reichert 2007; Moroz 2009). First, today's complex brains can be traced back to an ancestral central nervous system already present in a common ancestor of all bilaterian animals, the so-called "Urbilateria" (DeRobertis & Sasai 1996). Second, the origin of complex brains is independent among species in different lineages. According to the traditional view of animal evolution as a gradual progress from simple to complex, hitherto the hypothesis of a centralized ancestral nervous system in the "Urbilateria" followed by an essential reduction and losses in multiple Bilateria groups seemed highly unlikely (Moroz 2009). However, recent developmental studies reconstructing cell lineage phylogeny with molecular fingerprint tools have supported a single origin of the bilaterian nervous system (Tomer et al. 2010) suggesting that only one ancestral, albeit rather complex nervous system was at the origin of bilateral central nervous system evolution (Lichtneckert & Reichert 2005; Hirth & Reichert 2007). In protostomes the best studied higher brain centers are the mushroom bodies that are a typical feature of the arthropod brain. The recent molecular fingerprint studies revealed that the same patterns of genes are expressed during the ontogeny of arthropod and annelid mushroom bodies (Tomer et al. 2010), pointing towards an evolutionary relatedness of arthropod and annelid mushroom bodies. In addition to these developmental studies investigating gene expression patterns, a close morphological resemblance between the mushroom bodies in arthropods and annelids has also been proven by immunohistochemical analysis of the brain of a variety of polychaete annelids (Heuer 2010). In the light of the "new animal phylogeny" these results have raised the question whether these structures are indeed homologous and as a consequence whether mushroom body-like neuropils are present in other protostome clades as well. To address this question, the present thesis focused on the comparison of the internal brain anatomy of mollusks and nemerteans to that of arthropods and annelids.

Summarizing the results presented here, mushroom body neuropils that are typical for the arthropod and annelid brain could not been identified neither in the investigated molluscan taxa nor in the nemertean species. However, the nervous system of the investigated caudofoveate, scaphopod, and nemertean species exhibit clusters of cells that resemble the globuli cells that characterize the arthropod and annelid mushroom body. Repeated descriptions of small-diameter cells that are commonly referred to as globuli cells exist as well for the procerebrum of the gastropod mollusk *Helix pomatia* (Hanström 1928; Bullock & Horridge 1965; Bullock et al. 1977; Elekes & Nässel 1990; Elekes et al. 1993) and could also be confirmed by a recent study on the gastropod mollusk *Arion vulgaris* (personal observation). The procerebrum of pulmonate gastropods receives sensory input via the tentacle ganglion and thus is involved in the processing of olfactory information as secondary olfactory center (Chase 1986; Ratté & Chase 1997; Matsuo et al. 2009). As a

result the procerebrum of gastropods has been regarded as possible analogous to the mushroom bodies (Ratté & Chase 1997), even though the structure of the procerebrum differs apparently from that of the mushroom bodies. Besides the globuli-like cells in the procerebrum of gastropods, small globuli nerve cell cluster have also been described in the higher brain regions of cephalopods (Bullock et al. 1977). The brain of cephalopods is among the most complex of invertebrates, but the existence of globuli like-cells could not been confirmed by a more recent study by Wollesen et al. (2008). Descriptions of globuli-like cells in other molluscan taxa than gastropods and cephalopods are entirely lacking so far. In the present study globuli-like cells have been demonstrated in the posterior part of the brain of the investigated scaphopod species as well as in the precerebral ganglia of the caudofoveate species. In contrast, the central nervous system of the investigated solenogaster and polyplacophoran species do not possess globuli-like cells. In nemerteans so-called mushroom body forerunners have been described which form special clusters in the lateral parts of the dorsal lobes (Hanström 1928; Turner 1946; Bullock & Horridge 1965). These findings could not be confirmed by the present study. The nemertean species investigated here indeed exhibit globuli-like cells, but those cells were located in the cerebral organ rather than in the lateral parts of the dorsal lobes. However, these two findings are not necessarily in contrast, because none of the older descriptions refer to a specific species of Nemertea. In the majority of nemerteans such sophisticated cerebral organs as occurring in the investigated nemertean species *Lineus viridis* are not present. Thus in those species without defined cerebral organs a slightly different location is conceivable. Besides the descriptions of globuli-like cells in mollusks and nemerteans, such cells have also been described in one further lophotrochozoan phyla, the Platyhelminthes (Turner 1946; Bullock & Horridge 1965; Bullock et al. 1977; Keenan et al. 1981; Strausfeld et al. 1998). In the free living polyclad platyhelminth *Notoplana acticola* the globuli-like cells are arranged in two clusters that are situated anterior lateral to the brain (Keenan et al. 1981). As in nemerteans, these cell clusters have been considered as mushroom body forerunners (Keenan et al. 1981).

In the light of the "new animal phylogeny" on the one hand and the molecular fingerprint studies on the other hand, the absence of mushroom bodies in the investigated molluscan and nemertean species and the patchy distribution of globuli-like cells has to be interpreted as secondary character loss. A loss of mushroom body neuropils is as well described for representatives of arthropods (Andrew 2011) and annelids (Heuer 2010). In annelids for instance, mushroom bodies are primarily present in errant polychaetes, but have been lost in most other annelid taxa (Heuer 2010). However, according to Tomer et al. (2010) the evolutionary relatedness of arthropod and annelid mushroom bodies does not mean that a mushroom-body-like shape was already present in the protostome-deuterostome lineage. Rather, there was a less complex precursor structure and higher degrees of morphological complexity were obtained independently in different evolutionary lineages. Therefore in addition to the secondary loss scenario also an independent evolution of

mushroom body precursor structure is possible which may lead to major morphological differences. However, under similar environmental pressure these precursor structures may give rise to mushroom bodies in arthropods and annelids. In insects, species-specific ecology is actually reflected by morphological variability of mushroom bodies (Farris & Roberts 2005). For instance generalist feeders display larger and also more complex mushroom bodies than specialist feeders (Farris & Roberts 2005). Further possible scenarios leading to reduced brain complexity are the transition to sessile lifestyle or interstitial burrowing (Tomer et al. 2010). According to that, in annelids highest complexity of the mushroom bodies exists in errant and predatory polychaetes whereas infaunal or sedentary animals with a detritus or suspension feeding ecology possess no such higher brain centers (Heuer et al. 2010). The present study has demonstrated that a coherency of brain complexity and lifestyle does not apply for non-conchiferan mollusks where the brain of the burrowing and detritus feeding caudofoveates is most complex. Despite this difference there is one main uniting feature of the arthropod and annelid mushroom bodies to the structures that exhibit globuli-like cells in mollusks and nemerteans. In arthropods mushroom bodies play a role in olfactory processing as well as in associative learning and memory formation (Heisenberg 2003; Campbell & Turner 2010). Likewise the procerebrum of gastropods (Chase 1986; Ratté & Chase 1997; Matsuo et al. 2009), the precerebral organs of caudofoveates (Shigeno et al. 2007), and the cerebral organ of nemerteans (Ling 1969; Amerongen & Chia 1982; Ferraris 1985) are involved in the processing of olfactory respectively chemosensory information. The findings presented here therefore support the assumption of homologous precursor structures in the urbilaterian brain that give rise to more or less similar complex brain centers depending on the environmental pressure. In the case of the mushroom bodies and the structures exhibiting globuli-cells this environmental pressure seems to be related to the processing of olfactory information.

To draw final conclusions whether the globuli-like cells of mollusks and nemerteans are indeed evolutionary related to the globuli cells of the mushroom bodies in annelids and arthropods it would be enlightening to analyze the gene expression patterns of this structures via molecular fingerprint studies. Besides this promising approach that has already provided significant support for the homology of arthropod and annelid mushroom bodies, one further approach is the immunostaining with anti-DC0. DC0 is the catalytic subunit gene of protein kinase A that is preferentially expressed in the mushroom bodies of *Drosophila melanogaster* (Kalderon & Rubin 1988; Skoulakis et al. 1993). On the basis of this, the polyclonal anti-DC0 antibody was raised against the catalytic subunit of protein kinase A and has been shown to have a high affinity for globuli cell populations across a wide range of insect species (Farris & Strausfeld 2003; Farris et al. 2004; Farris 2005; Farris 2008a; Farris 2008b; Strausfeld et al. 2009; Farris & Schulmeister 2011; Farris et al. 2011). Given that anti-DC0 reliably labels globuli cell populations in insects it seems promising to test this antibody as well in non-arthropod taxa like mollusks and nemerteans that offer as well

globuli-like cells. However, first preliminary results show no specific labeling of anti-DC0 in the globuli cells of polychaete annelids (Heuer, personal communication).

Neuroanatomical glossary and data matrix – Essential steps toward a cladistic analysis of neuroanatomical data

Beyond retracing the evolution of individual brain centers, the main aim of those *Deep Metazoan Phylogeny* members investigating the nervous system was the reconstruction of the phylogenetic relationship among animals on the basis of neuroanatomical data. The most challenging step for a morphological based phylogenetic analysis is the partitioning of observed variation into different characters. Prerequisite for this purpose is a clear and consistent terminology of relevant morphological structures. Unfortunately, the long tradition in morphological research has led to a very inconsistent and confusing terminology. For example, the meaning of terms has changed over the years, the terminology used for morphological descriptions vary from author to author, various research interests have brought their own terminology, and morphological terms are often restricted to a certain taxon. In summary, up to now a standardized and commonly accepted morphological terminology is lacking (Vogt 2009; Vogt et al. 2010). Apart from the fact that no taxon-independent terminology exists, morphological descriptions are often not clearly separated from hypothetical conclusions, for example by using terms that imply homology of the described structure (Vogt 2009). The so called "linguistic problem of morphology" (Vogt et al. 2010) not only influences morphological descriptions, but also results in a weakness of morphological characters in phylogenetic analysis.

To overcome this problem the first step for the phylogenetic analysis based on neuroanatomical data was the generation of a glossary of invertebrate neuroanatomical terms and definitions. For the discussion which terms have to be included in such a glossary and also for the process of defining the individual terms, the members of the *Deep Metazoan Phylogeny* program, which are working on the nervous system, got together for several meetings within two years. With this glossary we provide a precise and consistent definition for 47 neuroanatomical terms. Many of the general terms are defined on the basis of Bullock and Horridge (1965), but as morphological research has continued since that publication revisions and updates were also necessary. Therefore each term is not only defined but rather consists of an additional explanatory background that also deals with the older literature. In order to ensure a comparability of distinct neuroanatomical structures between different taxa of invertebrates the neuroanatomical glossary is taxon-independent and free of homology assumptions. By using this consistent terminology for all descriptions of the anatomy of the nervous system, of course including the descriptions in the present thesis, the *Deep Metazoan Phylogeny* members enhance the comparability of defined structures between

various taxa and provide a basis for the construction of neuroanatomical characters for a morphological based phylogenetic analysis.

The first and crucial step of a cladistic analysis is the selection of terminal taxa and characters, which leads to the compilation of a data matrix (Jenner 2004). There are two alternative approaches concerning the selection of terminal taxa: the use of species (exemplar approach) versus the use of supraspecific taxa as terminal entities (Prendini 2001). Traditional many phylogenetic analyses based on morphology have used higher taxa as terminals by summarizing observations of a sample of species of one higher taxon into supraspecific terminals (Prendini 2001; Wiens 1998). In contrast, the exemplar approach involves the selection of a sample of species from each higher taxon, which are then scored as separate terminals (Prendini 2001). Since the use of exemplars is inevitable in molecular studies, the exemplar approach is exclusively qualified for the simultaneous analysis of morphological and molecular data. In addition, only the exemplar approach enables the addition of new taxa or characters in the future. Thus, for the compilation of a data matrix based on neuroanatomical data species were used as terminal taxa. So far a total of 122 species spanning all metazoan phyla are included in the neuroanatomical data matrix. In this regard the present study has contributed to the taxon sampling by providing the data for seven molluscan and one nemertean species.

The most challenging step for a morphological data matrix is the construction of characters, that is the partitioning of observed variation into discrete characters and character states (Wilkinson 1995). Characters can either be coded binary (absence/ presence) or as multistate and different coding decisions can lead to different phylogenetic trees. The bulk of characters in metazoan cladistic analysis are coded binary (Jenner 2004). However, this approach has been strongly criticized as the most problematic method for standard parsimony analysis (Jenner 2004), because it runs the risk that taxa may be united by the non-homology “absent” (Strong & Lipscomb 1999). Therefore, only one half of the current 128 characters from the neuroanatomical data matrix are coded as absent/ present and the other half are multistate characters. However, in both cases there is also the opportunity to code a character as inapplicable. Inapplicable character states occur when a character complex is transformed into multiple characters (Strong & Lipscomb 1999). If one taxa of the data matrix lacks the whole character complex, the multiple characters are inapplicable for this taxon (Strong & Lipscomb 1999). Previous cladistic studies have often struggled with incorrect character coding because of uncritically adopting character states from the literature without citing the source for each matrix entry (Jenner 2004). This turns detailed comparative morphological studies into an absolute requirement for carry out cladistic research. Thus, the neuroanatomical data matrix is in large parts grounded on own morphological investigations and descriptions of the *Deep Metazoan Phylogeny* members using a consistent terminology (see above).

In the light of the 128 characters and 122 taxa that are included in the neuroanatomical data matrix more than 15000 character states have to be coded. Up to now those *Deep Metazoan Phylogeny* members working on the data matrix got together for four meetings. In addition, also an independent however simultaneous coding is possible because of the implementation of the data matrix in an online database. Nevertheless, this time-consuming process is not yet completed. Therefore our main aim for the near future is the completion of the neuroanatomical data matrix. Followed by the phylogenetic computation the constructed data matrix will result in a phylogenetic tree that is exclusively based on neuroanatomical data. In comprising species of all metazoan phyla this tree can make valuable contributions to so far unsettled questions of phylogenetic relationship among animals.

5 Summary

Despite the plenty of molecular phylogenetic studies that emerged in recent years the position of major animal clades within the phylogenetic tree is still controversial. Therefore, the field of neurophylogeny that links the morphology of the nervous system to early evolutionary events emerged as a discipline that can be helpful for the inference of phylogenetic relationships as well as for the retracing of central nervous system evolution. So far, comparative neuroanatomical investigations in arthropods and annelids have suggested that certain brain centers are highly conserved during evolution. If structures like the mushroom bodies are indeed ancestral features of the bilaterian brain, we would expect to find similar neuropils in other invertebrate phyla as well. However, so far in depth studies of the detailed neuroanatomy of numerous invertebrate taxa are lacking. The present thesis therefore focuses on adding new comparative immunohistochemical data on the nervous system of the two lophotrochozoan phyla Mollusca and Nemertea.

The first part of the thesis presents an extensive survey on the detailed neuroanatomy of the lesser-known branches of the phylum Mollusca: Caudofoveata, Solenogastres, Polyplacophora, and Scaphopoda. By comparing the neuroanatomy of those different taxa it is demonstrated that the presence of two pairs of nerve cords of the medullary type is the main unifying feature of the three non-conchiferan taxa. In other respects, the great diversity of the phylum Mollusca likewise results in a considerable variability of the nervous system. Taking the results of both aplacophoran taxa, the brain as well as the nerve cords of Caudofoveata and Solenogastres are built in a conspicuous different manner. Structures that are similar in the nervous system of Caudofoveata and Solenogastres are as well present in the Polyplacophora. Thus a monophyly of Aplacophora could not be confirmed. Comparing the nervous system of the Caudofoveata, Solenogastres, and Polyplacophora, the caudofoveate brain seems to be most complex among the non-conchiferan taxa because it exclusively shows a division into discrete sections. The neuroanatomical data presented here, are therefore not in accordance with the Adenopoda hypothesis placing Caudofoveata basal within Mollusca.

The second part of the present thesis concentrates on the neuroanatomy of one representative of the phylum Nemertea. The nervous system of *Lineus viridis* basically

consists of a well developed brain that reveals no compartmentalized neuropils. Paired medullary cords emanate posteriorly from the ventral lobes of the brain while paired cerebral organs are posteriorly attached to the dorsal lobes of the brain. By presenting a detailed view on the peripheral nervous system as well, this study reveals that nemerteans possess four distinct but interconnected nerve plexus. The results indicate that a subepidermal plexus most likely belongs to the ground pattern of Lophotrochozoa.

Higher brain centers like the mushroom bodies that are typical for the arthropod and annelid brain could not been identified neither in the investigated molluscan taxa nor in the nemertean species. However, the nervous system of the investigated caudofoveate, scaphopod, and nemertean species exhibit clusters of cells resembling the globuli cells that characterize the arthropod and annelid mushroom body. Those globuli-like cell clusters are located in the precerebral ganglia of caudofoveates and the cerebral organs of nemerteans, respectively. Like the mushroom bodies of arthropods and annelids the precerebral ganglia as well as the cerebral organs are involved in the processing of olfactory information. Assuming that these cell clusters are indeed homologous, the present findings argue for the existence of mushroom body-like precursor structures in the last common ancestor of all protostomes.

Besides the contribution to the evolution of certain brain centers in protostomes, the neuroanatomical data obtained in the present study are used for the construction of a data matrix that will result in a phylogenetic tree that is exclusively based on neuroanatomical data.

6 Zusammenfassung

Die Verwandtschaftsverhältnisse zwischen bedeutenden Tiergruppen sind trotz einer Vielzahl an molekular phylogenetischen Untersuchungen nach wie vor ungeklärt. Neben der Molekularphylogenie hat daher in den letzten Jahren auch das Feld der Neurophylogenie an Bedeutung gewonnen. Vergleichende neuroanatomische Untersuchungen liefern nicht nur wichtige Merkmale für die Rekonstruktion von Verwandtschaftsverhältnissen, sondern können darüber hinaus auch genutzt werden um Rückschlüsse auf die Evolution des Nervensystems zu ziehen. Bisherige neuroanatomische Untersuchungen an Arthropoden und Anneliden lassen darauf schließen, dass bestimmte Gehirnzentren evolutiv hoch konserviert sind. Falls Strukturen wie die Pilzkörper tatsächlich ursprüngliche Merkmale des Gehirns der Bilateria sind, so sollten sie auch in anderen Invertebraten vorhanden sein. Detaillierte neuroanatomische Studien, wie sie für Arthropoden und Anneliden vorliegen, fehlen allerdings bislang für die meisten Invertebraten. Das Ziel der vorliegenden Arbeit besteht daher darin, neue vergleichende immunhistochemische Daten zum Nervensystem der Lophotrochozoenphyla Mollusca und Nemertea hinzuzufügen.

Das erste Kapitel der vorliegenden Arbeit ist eine detaillierte Untersuchung der Neuroanatomie der weniger bekannten Taxa des Phylums Mollusca: Caudofoveata, Solenogastres, Polyplacophora und Scaphopoda. Der Vergleich der Neuroanatomie dieser verschiedenen Taxa zeigt, dass bei den Caudofoveata, Solenogastres und Polyplacophora im Gegensatz zu den Conchifera zwei Paar Markstränge vorhanden sind. Ansonsten spiegelt sich die große Diversität des Phylums Mollusca auch in einer deutlichen Variabilität des Nervensystems wieder. So zeigen sich klare Unterschiede in der Architektur des Gehirn und der Nervenstränge beider Aplacophorentaxa. Auf der anderen Seite können Strukturen die in vergleichbarer Weise bei den Caudofoveata und Solenogastres vorhanden sind auch im Nervensystem der Polyplacophora nachgewiesen werden. Aufgrund dieser Ergebnisse kann die Monophylie der Aplacophora nicht bestätigt werden. Im Vergleich des Nervensystems der Caudofoveata, Solenogastres und Polyplacophora hat sich gezeigt, dass das Gehirn der Caudofoveata den größten Grad an Komplexität aufweist, da es als einziges in einzelne Abschnitte unterteilt ist. Die neuroanatomischen Daten der vorliegenden Arbeit stehen daher

im Widerspruch zu der Adenopodahypothese, welche die Caudofoveata als ursprünglichstes Molluskentaxon ansieht.

Das zweite Kapitel der vorliegenden Arbeit beschäftigt sich mit der Neuroanatomie eines Vertreters des Phylums Nemertea. Das Nervensystem von *Lineus viridis* besteht im Wesentlichen aus einem ausgeprägten Gehirn, welches nicht in einzelne Neuropile unterteilt ist. Paarige Markstränge gehen posterior von den ventralen Loben des Gehirns ab, während paarige Cerebralorgane posterior den dorsalen Loben des Gehirns anliegen. Neben der Beschreibung des zentralen Nervensystems liefert die vorliegende Studie auch eine detaillierte Darstellung des peripheren Nervensystems und zeigt so, dass Nemertea vier unterschiedliche miteinander in Verbindung stehende Nervenplexus aufweisen. Die Ergebnisse sprechen zudem dafür, dass ein subepidermaler Plexus zum Grundmuster der Lophotrochozoa gehört.

Höhere Gehirnzentren wie die Pilzkörper der Arthropoden und Anneliden konnten weder bei den verschiedenen Molluskentaxa noch bei der untersuchten Nemertinenart nachgewiesen werden. Das Nervensystem der untersuchten Caudofoveaten-, Scaphopoden- und Nemertinenarten weist allerdings Zellcluster auf, welche den für die Pilzkörper der Arthropoden und Anneliden charakteristischen Globulizellen ähneln. Die an Globulizellen erinnernden Zellcluster befinden sich bei den Caudofoveata in den Präcerebralganglien und bei den Nemertea in den Cerebralorganen. Wie die Pilzkörper der Arthropoda und Annelida sind die Präcerebralganglien wie auch die Cerebralorgane an der Verarbeitung olfaktorischer Informationen beteiligt. Geht man davon aus, dass diese Zellcluster tatsächlich homolog sind, so sprechen die vorliegenden Ergebnisse für die Existenz von pilzkörperähnlichen Vorstufen im letzten gemeinsamen Vorfahren aller Protostomia.

Die in der vorliegenden Arbeit gewonnen Daten tragen nicht nur zum Verständnis der Evolution von bestimmten Gehirnzentren der Protostomia bei, sondern gehen darüber hinaus in eine Datenmatrix ein, welche in einem auf neuroanatomischen Merkmalen basierenden Stammbaum resultiert.

7 References

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Katharina – Schön, dass Du das letzte Jahr meiner Arbeit hier warst. Ohne Dich wäre es wohl sehr viel einsamer gewesen… Ich drücke die Daumen, dass die Arbeitsgruppe demnächst doch noch zu neuen Doktoranden kommt, damit Du die nächsten Jahre nicht vereinsamst und auch jemanden hast, der Dir einen so tollen Hut bastelt!

Christiane – Vielen Dank, für die Einladung nach Norwegen! Trotz geringer Ausbeute war die Woche wirklich mein Exkursionshighlight. Vor allem danke ich Dir aber für die vielen Tiere, mit denen Du mich stets versorgt hast, und für Deinen Einsatz bei der Überarbeitung unseres gemeinsamen Manuskripts.

Pat – Danke für die nette Zusammenarbeit mit den Nemertinen!

Den Neurophylogenetikern des DMP – Schön, dass auf unseren zahlreichen Wochenendtreffen neben dem Glossar und der Matrix auch der Spaß nie zu kurz kam.

Marc – Es hat mich sehr gefreut, Dich während Deiner Staatsexamensarbeit zu betreuen und damit mal nicht die Einzige hier zu sein, die sich für Molluskengehirne interessiert...

Corinna – Nach der legendären Konferenz in Kopenhagen habe ich mich immer sehr darauf gefreut, wenn die nächste Tagung bevorstand und wir uns mal wieder ein Zimmer teilen oder ich mich einfach bei Dir in Hamburg einquartieren konnte. Viel Erfolg für Dein letztes Jahr und nochmal Danke für das Abschiedsbuch!

Flo & **Aleks** – Ich habe mich sehr gefreut, dass ihr extra zu meiner Prüfung angereist seid. Danke fürs Aufspüren der Bürgermeister-Neuropile.

Meinen Eltern – Ich danke euch sehr, dass ihr mich auf meinem Weg immer unterstützt.

Christian – Danke, dass Du jedes zweite Wochenende und auch sonst, wenn alles schief ging, jeder Zeit zu mir nach Aachen gekommen bist. Ohne Deine Unterstützung wäre das alles nicht möglich gewesen. Für mich hättest Du damit auch den Dr. verdient!

Lebenslauf

Persönliche Daten

Name	Simone Faller
Geburtstag	25.12.1981
Geburtsort	Frankfurt am Main
Staatsangehörigkeit	deutsch

Schulische und wissenschaftliche Ausbildung

08/1987 – 07/1992	Grundschule Weißkirchen
08/1992 – 05/2001	Gymnasium Oberursel
2001	Abschluss: Abitur
10/2001 – 09/2006	Studium der Biologie an der Goethe-Universität Frankfurt am Main
01/2006 – 09/2006	Diplomarbeit am Institut für Ökologie, Evolution und Diversität, Abteilung Phylogenie und Systematik, Goethe-Universität Frankfurt am Main
2006	Abschluss: Diplom-Biologin
11/2007 – 03/2012	Promotion am Institut für Biologie II, Abteilung für Entwicklungsbiologie und Morphologie der Tiere, RWTH Aachen University